

THE SPECIFICITY OF REINNERVATION
DURING MOTONEURON REGENERATION
IN THE COCKROACH Periplaneta Americana

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DECLARATION

David Young, Peter Snow and Peter Simmons gave their time and help in many discussions on scientific and technical problems.

I declare that all parts of this Thesis

describe my own original work.

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I owe a special debt to Gill Holmes and Keith and Mary Williams who gave friendship and endured my presence for four difficult months.

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ABSTRACT

The anatomy and physiological connections of some identified motoneurons in the mesothoracic ganglion of the cockroach *Periplaneta americana* are described.

Several identified motoneurons make functional connections with foreign muscles when regenerating to a leg from which the coxal depressor muscle "Never be certain of anything" pattern of innervation found suggest that there are restrictions on which muscles a given motoneuron may It's a sign of weakness."

The pattern of axonal branching and the physiological connections of motoneurons regenerating to an intact leg after crushing nerve 5 have been investigated. The axons of motoneurons 30 and D₂ branch repeatedly distal to the crush site. Dr. Who to Leela. axonal branches reach nerve 5 in every neuron examined. The normal coxal depressor motoneurons 30, D₂, 40 and 44 invariably send axons down nerve 5 as determined by the cobalt technique. A number of foreign nerve 5 neurons also send axons down nerve 5; different neurons fill at different frequencies but in no case does a foreign neuron fill in all preparations. The number of foreign neurons filled decreases with increasing post-operative time.

Motoneurons 30 and D₂ make functional connections with inappropriate nerve 5 coxal depressor muscles following nerve 5 crush. The nerve 5 coxal depressors are also innervated by a number of unidentified excitatory motoneurons other than the normal motoneurons 30 and D₂. In some cases the excitatory junction potentials elicited by these "foreign" motoneurons could be correlated with nerve 5 spikes. Muscles are often simultaneously innervated by both appropriate and inappropriate motoneurons.

Several observations indicate that innervation is not random: regenerating motoneurons rarely innervate muscles which have not been previously denervated; the number of neurons which reinnervate a

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Several identified motoneurons make functional connections with foreign muscles when regenerating to a leg from which the coxal depressor muscles have been excised. The patterns of innervation found suggest that there are restrictions on which muscles a given motoneuron may innervate.

The pattern of axonal branching and the physiological connections of motoneurons regenerating to an intact leg after crushing nerve 5 have been investigated. The axons of motoneurons 30 and D₅ branch repeatedly distal to the crush site: at least two axonal branches reach nerve 5rl in every neuron examined. The normal coxal depressor motoneurons 30, D₅, 40 and 44 invariably send axons down nerve 5rl as determined by the cobalt backfill technique. A number of foreign nerve 5 neurons also send axons down nerve 5rl: different neurons fill at different frequencies but in no case does a foreign neuron fill in all preparations. The number of foreign neurons filled decreases with increasing post-operative times.

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Several observations indicate that innervation is not random: regenerating motoneurons rarely innervate muscles which have not been previously denervated; the number of neurons which reinnervate a

given muscle at one time is limited; motoneuron 30 rarely innervates femoral or tibial muscles; inhibitory motoneurons appear not to innervate inappropriate muscles.

The frequency of occurrence of functional misinnervation decreases with increasing post-operative time. However, some inappropriate functional connections are found at the latest post-operative times examined (seven months).

The above results are discussed in relation to two questions:

- 1) When are crucial decisions made during neuronal development?, and
- 2) What are the rules for axon growth and functional synapse formation?

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1.1 THE PROBLEM: HOW DOES SPECIFIC CONNECTIVITY ARISE?

Development is characterized by the emergence of diversity and order. These processes are especially obvious in the development of the nervous system. Diversity is expressed in the appearance, from the embryo to the adult, of a great variety of nerve cells, each type possessing a unique combination of cellular properties. Order is illustrated by one of these properties: the complex yet highly specific pattern of synaptic connections between nerve cells and their targets. This particular property has come to be known as "neuronal specificity".

Biologists began to ask how these orderly patterns of connections arise as soon as their existence was revealed. In 1937 Ramon y Cajal wondered "What mysterious forces stimulate the . . . migration of the cells and fibres in predetermined directions as if in obedience to a skillfully arranged plan and finally establish these protoplasmic kisses?" (Ramon y Cajal, 1937). To this day we remain ignorant of the developmental forces which generate specific connectivity in the nervous system.

CHAPTER 1

INTRODUCTION

1.2 ROLE OF EXPERIENCE VERSUS GENES

A first approach to this problem is to ask whether the pattern of connections between neurons is determined solely by a predetermined genetic plan lying within the body cells of the organism, or whether factors in the embryonic environment can affect or direct which connections are formed. There is a large body of literature arguing for experiential effects in the development of behaviour (reviewed in Gottlieb, 1976). In many cases these arguments are based upon the finding that abnormal patterns of behaviour result when animals are reared in abnormal environments (see Gottlieb, 1976, pp. 47-49). While such experiments illustrate the plasticity of the nervous system, they do not necessarily prove that environmental effects are important in the development of normal behaviour. Furthermore, in those cases where experiential effects have been implicated in the development of specific behavioural patterns, it is generally unclear as to (a) which neuronal

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pathways are being influenced and (b) whether the environmental factors direct the formation of completely new pathways or merely stabilise pre-existing pathways. Three systems in which we have some knowledge at the neuronal level of the effect of experiential factors can be cited:

- a) Kitten cortical neurons fail to respond to visual stimulation of the eye which normally drives them if that eye is closed during the first three months of life (Wiesel and Hubel, 1965). Such animals are behaviourally "blind" in the operated eye. (Wiesel and Hubel, 1963.)
- b) The pattern of neuronal connections linking the two optic tecta in Xenopus laevis is less organised in dark-reared animals than in control animals reared in a normal light-dark cycle, and is not modified by rotation of the eye prior to stage 58, as is the pattern in control animals (Keating and Feldman, 1975).
- c) The sensitivity of the adult cricket medial giant interneuron to sensory input from cercal sensory neurons is much reduced compared to controls if the neural activity in these sensory neurons is blocked during development (Matsumoto and Murphey, 1977).

In contrast, several lines of evidence suggest that, at least in some species, the "wiring" of parts of the nervous system takes place largely under genetic control. Such evidence includes the following:

- a) Adult crickets produce the calling song characteristic of their own species regardless of their acoustical experience during development. Genetic crosses between crickets of different species produce hybrids which give unique calling songs (Bentley and Hoy, 1974).
- b) Functionally maladaptive behaviours, produced, for example, by grafting a left supernumerary forelimb onto the right flank of a salamander (Weiss, 1937) or by rotating the eye of an adult salamander through various angles (Sperry, 1951), are not corrected for with time.
- c) Adult or adult-like motor patterns can be produced by immature insects; e.g. orthopteran nymphs (Bentley and Hoy, 1970; Altman and Tyrer, 1974) and lepidopteran pupae (Kammer and Rheuben, 1976). The development of motor output patterns does not

seem to be dependent upon feedback from the peripheral muscle or sense organs which normally monitor the results of the motor activity, since some of these organs are not fully functional in nymphs or pupae.

d) The number, anatomy and physiology of identified invertebrate neurons in isogenic animals are highly stereotyped (Goodman, 1977; Goodman and Heitler, 1977; Macagno et al., 1973). Some variability does exist which cannot be explained by differences in genotype, but neither can such variability be easily ascribed to environmental factors. (Macagno et al., 1973).

In some systems then, assembly of neuronal circuits takes place largely or solely under genetic control. Where experiential factors have been implicated it seems likely that they modify or stabilise a basic pattern which is laid down according to genetic instructions.

1.3 DEVELOPMENT OF THE "CHEMO-AFFINITY" HYPOTHESIS

1.3.1 PERIPHERAL SPECIFICATION VERSUS NEURONAL IDENTITY

Given that the pattern of neuronal connections is largely "preprogrammed" within the body cells of the animal, we may ask whether individual neurons are preprogrammed to innervate their future targets. An alternative view is that the growing neuron is initially "anonymous"; it innervates a target cell at random, then acquires information from that cell which specifies its connections with other neurons (and hence its physiological identity). This latter concept, known as "peripheral specification" was developed in the 1930's largely as a result of Weiss's studies in the salamander. Weiss found that supernumerary limbs transplanted to abnormal sites on the flank of an adult salamander developed, upon reinnervation, movements which were co-ordinated with their homologous limbs, even though these supernumerary limbs appeared to be innervated by an abnormal nerve supply (Weiss, 1936). He suggested that the muscles in the supernumerary limb had "impressed their character" upon the foreign nerves,

respecifying their central connections in such a way that co-ordinated movements resulted. More recent work, however, has shown that while gross morphological criteria may suggest that muscles are misinnervated, when physiological criteria are applied, the same muscles appear to be functionally innervated only by their correct nerves (Grimm, 1971). (This example illustrates what has been a widespread problem in regeneration studies; unambiguously establishing which neurons innervate which targets.) At this stage it seems unnecessary to invoke "peripheral specification" to explain restoration of appropriate function during motoneuron regeneration. In the absence of direct anatomical and physiological evidence that the central connections of motor and sensory neurons change when those neurons are forced to innervate a foreign target and that such changes are appropriate to the new target, we must consider the theory of "peripheral specification" unproven.

1.3.2 SPECIFIC REINNERVATION AT THE LEVEL OF WHOLE NERVES

Sperry and Arora (1965) and Mark (1965) tested the theory of peripheral specification as an explanation for the recovery of co-ordinated movement in extraocular muscles in the fish and in fish fin muscles, respectively, after nerve section. These workers forced nerves to innervate foreign muscles to see whether the timing of contractions elicited by a misplaced nerve changed to the type found in the nerve which normally innervates the muscle. In neither case was such an effect observed: rather the contractions of cross-innervated muscles tended to be timed appropriately for the original muscle from which the crossed nerve branch had been taken. Furthermore "there appeared to be a definite tendency for regenerating nerves to restore normal nerve-muscle combinations even though the latter were strongly disfavoured by the mechanical conditions". (Sperry and Arora, 1965). These results suggest that the recovery of function seen in these systems after regeneration of cut nerves can best be explained by selective regrowth of nerves to their original muscles.

It has since been convincingly demonstrated in many other systems that recovery of normal function during regeneration in adults is achieved by a specific regrowth of nerves to their original targets. Examples include:

- a) Specific reinnervation of muscles in amphibia (Cass et al., 1973) and birds (Feng, Wu and Yang, 1965) by regenerating motor nerves.
- b) Specific reinnervation of peripheral targets by sensory nerves in amphibia (Langboner and Venable, 1974) and cats (Burgess et al., 1974) and of central targets by regenerating sensory nerves in amphibia (Gaze, 1970), and birds (Landmesser and Pilar, 1970).

The phenomenon of specific reinnervation suggests that adult neurons possess some intrinsic property which enables them to select their correct target from amongst a large number of potential target cells. Sperry extrapolated these findings to the embryo in an attempt to explain how neurons come to innervate their correct targets during development (Sperry, 1944). This hypothesis, known as the "Chemo-Affinity" theory, is presently the most widely favoured explanation for the phenomenon of neuronal specificity. Sperry proposed that during development a neuron and its future target cell acquire complementary cytochemical markers. These labels are acquired, he claimed, by the common embryological processes of cell determination and differentiation which are taking place concurrently in other body cells. Specific connectivity between pre- and post-synaptic cells is achieved later in development by a "matching" of these complementary labels. It bears emphasising that Sperry developed his theory from observations on regenerating adult neurons, not embryonic neurons themselves. To date we have no direct evidence that the mechanisms for the establishment of specific connections are the same in the embryo and the adult. Indeed there are obvious differences between the embryonic and adult neural environments which may demand different strategies for achieving specific connectivity. Such differences include:

- a) The degree to which pre-synaptic cells can determine or change

the properties of their post-synaptic targets (or vice-versa) probably differs in the embryo and the adult. Embryonic cells would be expected to show more plasticity as they may not be fully determined. Thus, failure to demonstrate effects such as "peripheral specification" (see section 1.3.1) or "neurotrophic determination" (see section 1.4.1) in the adult, does not preclude the possibility that such mechanisms operate in the embryo.

b) After cutting a nerve and allowing it to regenerate, several different neurons are presented with the opportunity of innervating a given target at the same time; and conversely a given neuron may have access to many possible targets. In the embryo, however, both axon outgrowth and the appearance of target cells may be sequential.

Hence spatiotemporal mechanisms for generating specific connectivity seem unlikely to operate in the adult but are a distinct possibility in the embryo.

c) A regenerating neuron may be guided to its correct target cell by following the distal glial sheath. Such an option is not available to an embryonic neuron sending out its axon into "uncharted territory". Embryonic axons may, however, follow the path of axons which precede them in development (see section 5.3.1).

d) The distances between neurons and their future targets are much greater in the adult than in the embryo while embryonic targets are much smaller than adult targets. Hence neurons may use different strategies to reach their targets in the two situations.

Despite these differences it has been, and continues to be, assumed that knowledge of what neurons are capable of doing during regeneration may shed light on what they do during development. Sooner or later this assumption will have to be tested by looking directly at neurons in the embryo. However, because of the limited opportunities for experimental studies in the embryo (for reasons of small size and inaccessibility), most studies will undoubtedly continue to focus on regeneration.

1.3.3 SPECIFIC REINNERVATION BY INDIVIDUAL NEURONS

The examples of specific reinnervation presented above show that whole nerves or small groups of neurons can reconnect specifically with their original targets after regeneration. The level of specificity required to generate the pattern of neuronal connections seen in the normal mature animal is much greater than this: individual neurons must be capable of reforming specific connections with their original post-synaptic targets. This has not been demonstrated in the vertebrates due to the paucity of cases where one can reliably identify the same neuron in different individuals. Identifiable neurons are, however, abundant in the invertebrates. Within a given species one can reliably identify individual motor, sensory and interneurons in different animals by the anatomical features of soma size and position and dendritic branching pattern (e.g. Davis, 1970; Young, 1969). Individual neurons identified by these criteria are found to have a stereotyped pattern of central and peripheral synaptic connections (e.g. Nicholls and Baylor, 1968).

A number of studies have shown that identified invertebrate neurons are capable of reestablishing connections with their original targets during regeneration. For example:

- a) Pearson and Bradley (1972) find in the metathoracic ganglion of the cockroach that the fast excitatory motoneuron to the coxal depressor muscles reconnects specifically with these muscles after axotomy.
- b) Van Essen and Jansen (1976) report specific regeneration of identified leech sensory neurons to their correct skin areas after nerve crush.
- c) Carbonetto and Muller (1977) find that "S-cell" interneurons in the leech ventral nerve cord reconnect specifically with their appropriate target neurons (other S-cell interneurons) after nerve crush. These results support the central concept in Sperry's "Chemo-Affinity" theory, namely that individual neurons are prelabelled in some way which enables them to recognise their appropriate post-synaptic targets.

While most of the available experimental evidence is in accord with this theory we have no direct proof of the existence of such labels. Their chemical nature can only be guessed at and it is unclear how they could be used to form a stable pattern of synaptic connections between pre- and post-synaptic cells. We have also only begun to consider how a neuron may acquire such labels during development. If we can consider the property of neuronal specificity to be like any other differentiated cell property, then these questions can be seen as equivalent to the classical embryological problems of the nature, expression and acquisition of the differentiated state. We may hope that concepts derived from classical embryological studies will throw light on the problem of neuronal specificity.

1.4 COMPETING THEORIES

While Sperry's "Chemo-Affinity" theory is widely favoured it is far from being universally accepted. At least four groups of theories challenge it as an explanation for the genesis of specific connectivity during development. One of these theories, peripheral specification, has already been considered in section 1.3.1. The other theories can be grouped under the headings "Neurotrophic Determination", "Spatiotemporal Order" and "Systems Matching".

1.4.1 NEUROTROPHIC DETERMINATION

This theory proposes that differentiated properties of the post-synaptic cell (e.g. membrane properties, transmitter synthesis, position in body, neuro-anatomy) are specified through an inductive interaction with the pre-synaptic neuron. (See Atwood, 1973). Recognition of a specific post-synaptic cell by the pre-synaptic neuron is not required by this mechanism: any cell capable of responding to the pre-synaptic neuron is an appropriate target.

Several lines of evidence have been advanced to support this theory. First, it has been shown in several systems that an intact pre-synaptic nerve supply is essential for the maintenance of mature

post-synaptic cellular properties, (reviewed in Smith and Kreutzberg, 1975; Guth, 1974). A possible determinative, rather than merely maintaining role for pre-synaptic neurons is indicated by the observation that the physiological properties of mammalian muscle cells may be altered after cross reinnervation with a foreign nerve (reviewed in Close, 1972, pp. 170-177). These demonstrations of neurotrophic effects in the adult beg the question as to whether similar phenomena occur during development. We have no direct evidence on this latter point, although Sisto Daneo and Filigamo (1975) find that the first differences in the pattern of differentiation of "fast" and "slow" fibres of chick embryonic eye muscle coincide with the first signs of innervation in these fibres. It is dangerous to infer a causal link between these two events on the basis of descriptive evidence alone. Even if more substantial evidence in support of neurotrophic effects of this type during embryogenesis were available, we would be far removed from the conclusion that such trophic effects determine the whole differentiated character of the post-synaptic cell, as the "Neurotrophic Determination" theory requires.

1.4.2 SPATIOTEMPORAL ORDER

Another alternative theory, the "Spatiotemporal Order" model (Jacobson, 1970, pp. 328-329) proposes that the stereotyped pattern of neuronal connections seen in the adult results from the ordering in space and time of sequences of pre-synaptic axon outgrowth and post-synaptic cell differentiation such that the first cell contacted by a growing axon happens to be the appropriate target for that neuron. This mechanism, like "Neurotrophic Determination", dispenses with the need for specific recognition between pre- and post-synaptic cells. There may, however, be a requirement to distinguish between a particular class of cells and other embryonic cells. Furthermore, this model requires that the outgrowing pre-synaptic axon be guided in a particular direction. There are at least two ways in which this directed axon outgrowth could be achieved which do not depend upon the

pre-synaptic axon tip being guided by cues specific to its future target. (We must reject the possibility of guidance by specific cues as it implies that the pre-synaptic neuron is "marked" so as to recognise specific features of the post-synaptic cell. See section 5.3.1). These two mechanisms are:

- a) The direction of axon outgrowth is decided by instructions entirely within the pre-synaptic neuron, i.e. the axon is growing out "blind" to its environment. As a precedent, Van der Loos (1965) has shown that cat pyramidal neurons initially send out axons in a constant orientation with respect to the cell body, irrespective of the orientation of the soma in the tissue.
- b) The axon is passively guided by non-specific features in the embryonic landscape. This path (which may correspond to the path of least physical resistance) just "happens" to lead the axon to its appropriate target. It is, in fact, known that growing neurons in tissue culture preferentially follow ordered arrays of collagen filaments (Weiss, 1934).

Lopresti et al. (1973) claim that a "Spatiotemporal Order" model can explain the development of ordered connections between optic nerve fibres and optic lamina neuroblasts in Daphnia. Their model, based on descriptive studies of synaptogenesis, assumes the following:

- a) axon outgrowth from receptor neurons is triggered by contact with a landmark in the midplane of the embryonic eye,
- b) the axon follows the midplane as it grows towards the optic lamina,
- c) any morphologically undifferentiated neuroblast can act as a target for any axon i.e. the outgrowing axon innervates the first undifferentiated neuroblast it happens to meet,
- d) a stereotyped response, namely movement of the optic cartridge away from the midline, ensues when a given optic axon has contacted five undifferentiated neuroblasts (cf. neurotrophic determination, section 1.4.1).

Similarly Meinertzhagen (1972) concludes from a study of errors

in retinula axon-lamina cell connections in adult Calliphora that retinula cells tend to grow in the direction of their future target lamina neurons and that the axon grows over a fixed distance. If there is an error in either direction or degree of axon growth the neuron will terminate on an inappropriate lamina neuron. It innervates that incorrect cell regardless, showing that it is not pre-labelled to recognise a specific lamina cell.

A "Spatiotemporal Order" mechanism would seem to economise on the amount of genetic information needed to construct specific patterns of neuronal connections as only very general instructions such as, "follow the midline", "innervate the first undifferentiated neuroblast you meet", need be coded within neurons. It is expected that such a mechanism may be used to generate specific connections between very large populations of neurons, as in the invertebrate visual system. However, there is some experimental evidence to suggest that this mechanism does not have universal application. The "inside-out" order of neuron differentiation in normal mice (in the sense that neurons generated late in development attain more superficial positions in the cortex than neurons generated earlier) is reversed in "reeler" mutant mice (Caviness, 1977). Despite this reversal of the spatiotemporal developmental order, reeler neurons appear to make normal connections within the cortex (Caviness, 1977). Hunt and Jacobson (1972) delayed the arrival of optic fibres in the frog optic tectum by transplanting an early embryonic eye to the side of another embryo and leaving it there for 30 days. During this time many new cells were added to the transplanted retina and the original tectum. Nevertheless when the eye was replaced in its original socket and allowed to reform connections with the brain for the first time, a normal retinotectal projection developed.

1.4.3 "SYSTEMS MATCHING" HYPOTHESIS

A final group of hypotheses, going under the title of "Systems Matching" (Gaze and Keating, 1972), has been developed to account

for several observations in the lower vertebrate retino-tectal system which argue against a rigid "Chemo-Affinity" model. These observations are:

- a) The modes of growth of the retina and optic tectum are such that connections formed early in the development of these organs would need to be "shifted" later to produce the adult pattern of connections (Gaze et al., 1974). Scott and Lazar (1976) have recently demonstrated histologically that a given optic nerve fibre arborizes consecutively at a series of tectal sites during development. Continual shifting of synaptic connections seems to be incompatible with a fixed specificity model.
- b) Following excision and transplantation experiments which alter the normal size relationship between the retina and optic tectum, the so-called "size disparity" paradigm, retinal neurons may innervate inappropriate regions of the optic tectum (see Gaze, 1970).

Meyer and Sperry (1976) have attempted to reconcile the size disparity findings with Sperry's original hypothesis, by proposing that the natural affinity between a retinal neuron and its appropriate tectal region is overridden by the attractive influence of nearby denervated tectal neurons.

Others, however, have abandoned the idea of fixed locus affinities in favour of a "Systems Matching" type hypothesis in which matching takes place not between individual retinal and tectal neurons but between tectal and retinal "systems" (Gaze and Keating, 1972). The phenomenon which these hypotheses seek to explain is that the "connections formed by a given retinal ganglion cell depend not only on the retinal situation of that ganglion cell but also on the extent of the retinal fibre complement projecting to the tectum and the extent of tectum available" (Gaze and Keating, 1972). "Systems Matching" is merely a statement of this phenomenon and is not in itself a mechanistic explanation of the phenomenon. Hope et al. (1976) have constructed an explicit cellular model based on the "Systems Matching" concept. They hypothesize that retinal fibres can tell, from local

information, the directions rostral and medial in the tectum (for example from the slope of chemical concentration gradients along the rostral/caudal and lateral/medial axes) and also the relative locations of their cell bodies in the retina. A retinal fibre adjusts its position in the tectum so that its terminal is caudal to the tectal terminals of all other retinal fibres whose cell bodies are located nasal to its cell body in the retina.

Similar models have been advanced by Prestige and Wilshaw (1975) and Von der Malsburg and Wilshaw (1977) with the difference that these workers argue for selection of appropriate synapses by stabilisation of existing synapses rather than choice prior to synaptogenesis (see sections 5.2.2 and 5.2.3). In none of these "Systems Matching" hypotheses is there a requirement for "matching" between specific pre- and post-synaptic cells, as is the case in the "Chemo-Affinity" theory.

Certainly the degree of neuronal plasticity revealed in "size disparity" experiments casts doubt on the validity of the "Chemo-Affinity" hypothesis in its rigid "one neuron-to-one target" form. But in view of our lack of understanding of the cellular events underlying such phenomena it would seem premature to totally reject the "Chemo-Affinity" hypothesis in favour of a "Systems Matching" hypothesis at this stage (see Hunt and Jacobson, 1974).

1.5 RULES FOR CONNECTIVITY

If each synapse in the nervous system owes its existence to the presence of a unique cytochemical label on pre- and post-synaptic cells, then we require some 10^{10} - 10^{12} genes to code for all of the synapses in the human brain. Since there are, at the maximum, 10^6 genes in the human genome, there must exist "gene saving" mechanisms to reduce the amount of information needed to generate the mature pattern of synaptic interconnections (Horridge, 1968, p. 325).

Two possible mechanisms are:

- a) Share the same label for several different combinations of pre-

and post-synaptic cells in situations where confusion is unlikely to occur because of spatiotemporal restrictions on innervation.

b) Use a limited number of chemical labels in different proportions to generate unique combinations and specify each synapse by a different combination. (This mechanism will "save" on structural genes but may require many regulatory genes).

If either strategy is used, a given pre-synaptic neuron should have a number of potential post-synaptic targets which it does not innervate in the normal animal but may innervate in animals with altered neuroanatomy. That is, the rules for connectivity are more complex than "a neuron may innervate only its normal target cells". Information about the range of potential connections and the circumstances under which these will form should shed light on the rules governing the formation of normal neural connections.

Two methods have been used to alter normal neuroanatomy and hence to expose neurons to potential new targets. The first of these is surgical: crush, cut, interchange or deflect nerves; remove, graft or transplant post-synaptic targets to new sites.

A general rule which has emerged from such studies in a wide range of animals is that neurons are capable of innervating targets homologous to their normal ones on the opposite side of the body. In the invertebrates: cockroach coxal depressor motoneurons innervate contralateral coxal depressor muscles (Fourtner et al., 1977) and cricket cercal sensory neurons may form excitatory synaptic connections on giant interneurons contralateral to their normal targets (Palka and Schubiger, 1975). In the vertebrates: "granule cells of the rat dentate gyrus which are denervated by unilateral destruction of the entorhinal cortex are reinnervated in part by proliferation of surviving pathways from the contralateral entorhinal cortex" (Steward, 1976); after unilateral enucleation in neonatal rats, neurons from the remaining eye project in topographic order to subcortical visual centres (Lund et al., 1973). An ipsilateral pathway exists in normal animals but is less developed than the experimentally

produced projection.

These results suggest that the rules for connectivity are repeated on either side of the body. Whether left hand side neurons are identical to their right hand side homologues can be tested by allowing correct neurons to regrow to their targets. If right hand side motoneurons displace left hand side motoneurons from right hand side muscles we may conclude that left hand side and right hand side neurons are not identical.

Similarly Young's (1972) finding that an identified mesothoracic motoneuron in the cockroach specifically innervates metathoracic muscles homologous to its normal mesothoracic target muscles, suggests that connectivity rules are also repeated segmentally.

In general, however, catalogues of permissible aberrant connections (for a given group of pre- and post-synaptic neurons) are not complete enough to allow us to define connectivity rules. One problem has been determining the exact identity of the pre- and post-synaptic neurons participating in the aberrant connections (Steward, 1976). The invertebrates would seem to offer a powerful lever in this type of study.

An alternative approach to producing aberrant connections is the use of neurological mutants. Unfortunately the mutants investigated to date have yielded little information as specificity of innervation seems to be preserved even though neurons are displaced (e.g. "reeler" mutant in mice, Caviness, 1977) or duplicated (e.g. Kuffler and Muller, 1974; Triestman and Schwartz, 1976).

Both the surgical and genetic approaches to the problem of connectivity rules assume that a neuron which forms an aberrant connection possesses the same "identity" in the experimental animal as in the normal animal. This assumption may not be justified; it is quite possible, especially in the case of mutants, that the aberrant neuron has acquired different cytochemical labels such that it is no longer recognised as being foreign by its new target. It is difficult to control for this possibility; one approach is to challenge the neuron to

innervate its normal target (e.g. Lund and Lund, 1976; Feldman et al., 1975; Schmidt, 1978).

1.6 ACQUISITION OF NEURONAL IDENTITY

We may consider the problem of how a neuron acquires that unique, intrinsic property which enables it to seek out and recognise a specific target cell, namely, neuronal "identity", in the same light as the general embryological problems of cell determination and differentiation. Indeed the approach to this problem in the nervous system is founded upon classical embryological concepts. The parallel extends further: we know as little about how neuronal identity is acquired as how other differentiated cell properties are determined.

The transplantation experiments of Jacobson and Hunt in the amphibian retinotectal system reveal a critical five-hour period in early development during which the reference axes, around which the future pattern of locus specificities will be organised, are specified (Jacobson, 1970, p. 321). As might be expected, however, there are no obvious changes in the retinal neurons during this period which might shed light on the nature of the specification process. One correlation which can be drawn is that specification occurs immediately after the cessation of DNA synthesis in retinal cells (Jacobson, 1970, p. 324). Whether a causal relationship exists between these events is unknown.

There is much evidence to suggest that the developmental fate of embryonic cells in general is determined by their lineage (e.g. Dienstman and Holtzer, 1975). We do not yet have any direct evidence that the lineage of a neuron determines its specified state. Some observations are compatible with this concept, however. Supernumerary leech (Kuffler and Muller, 1974) and Aplysia (Triestman and Schwartz, 1976) neurons have the same connectivity patterns as their normal partners. (This is an argument for a role for cell lineage only if it is assumed that the original and duplicated neurons arise from

identical cell lines.) Prolonging the cell cycle or altering the micro-environment are insufficient conditions to cause retinal neural precursor cells to directly generate mature retinal neurons. Rather it seems that presumptive neurons need to pass through a certain sequence of divisions before they can generate fully differentiated neurons (Hunt, 1975). There is an exact correlation between ancestry and final functional class in nematode neurons (White et al., 1976), suggesting that the fate of individual cells in this animal may be determined by their ancestry. The studies initiated by Bate (1976a) in locusts may provide a tool for testing the importance of cell lineage in determining neuronal connectivity.

An alternative view, which has also received much consideration in the classical embryological literature, is that the fate of a cell is determined by positional information received by that cell (Wolpert, 1971). This information may take the form of the local concentration of a specific morphogen in a gradient of that chemical. Again, however, we have no direct experimental evidence that the synaptic connections formed by a neuron are determined by positional information, although some suggestive data are available. Bate (1973) described a group of sensory hairs associated with gin traps on abdominal segments of privet hawkmoth pupae which, when stimulated, elicit a closure of adjacent gin traps. The hairs which evoke this response all lie at the same anteroposterior level as the gin trap, although not necessarily within the trap itself: sensory cells anterior or posterior to this level do not participate in the reflex. Bate and Lawrence (1973) interpret this as an indication that an anteroposterior gradient determines central connections of these receptor neurons. Palka and Schubiger (1975) find two populations of filiform hairs on cricket cerci. These groups differ morphologically only in the orientation of their sockets, one being transverse to the main cercal axis (T-hairs), the other being longitudinal (L-hairs). T-hairs, which are located on the dorsal and ventral cercal surfaces, excite giant interneurons in the ventral nerve cord strongly, whereas

L-hairs, which are located on the lateral and medial surfaces, excite these interneurons only weakly. The pattern of sensory input is preserved even when cerci are rotated, suggesting that T-hairs are specifically labelled to recognise and synapse with giant interneurons. Edwards and Palka (1976) propose that T-hairs acquire this label from a gradient of positional information, whose presence is inferred from the phenomena of back rotation and supernumerary formation following cercal rotation.

Gradients of positional information have also been invoked to explain formation of topographically ordered retino-tectal projections in fish and amphibia (see "Systems Matching" section 1.4.3). In these models, however, positional information is thought to enable a neuron to determine its rank order in an array of neurons but does not directly specify the connections of that neuron by a "labelling" method.

Experimental tests of the cell lineage and positional information hypotheses may be possible in the future. Clearly, however, we require a population of pre- and post-synaptic cells whose connections are known at the single cell level before we can apply such tests.

1.7 DECISION POINTS DURING NEURONAL DEVELOPMENT

The issue which most workers in the field of developmental neurobiology have been, and continue to be, concerned with is whether or not specific connectivity results from the matching of pre- and post-synaptic cells with specific chemical affinities. At present, however, we have no direct evidence for the existence of such affinities, let alone an understanding of how they generate specific connectivity. Before we can hope to approach these problems we need to discover when, during neuronal development, the crucial decisions leading to the formation of stable, functional synapses are made. The term "neuronal development" is here used in a restricted sense to refer to that sequence of developmental events from the first stages of axon

outgrowth to the formation of a stable synapse (fig. 1). By analogy with current views of how development occurs in other systems, it seems reasonable to suppose that there exist "gates" through which a neuron must pass if it is to continue to progress down this developmental sequence: these gates represent the decision points. Several possible candidates for gating points are indicated in fig. 1. Neurons fail to form stable synapses with inappropriate muscles because they are blocked at a gate; which gate may depend upon the pre-synaptic/post-synaptic neuron combination in question. For example neuron A may be blocked in stage 1 when growing to target B' but in stage 2 when growing to target C'.

To quote Horridge (1972): "We must define the times and number of choices which are available to a neuron, then observe by its own cellular behaviour that relevant differences exist for it". To date, little attention has been given to this question of the important decision making points during neuronal development.

1.8 THE APPROACH TAKEN IN THIS STUDY

Some aspects of the problem of specific connectivity, for example, the questions of the existence, nature and acquisition of specific chemical affinities, seem beyond a direct experimental approach at this stage. There would seem to be some hope of making headway on at least two fronts, however:

- a) in determining the times during neuronal development when crucial decisions are made;
- b) in elucidating the rules for connectivity from the pattern of aberrant connections made by a neuron in unusual experimental situations.

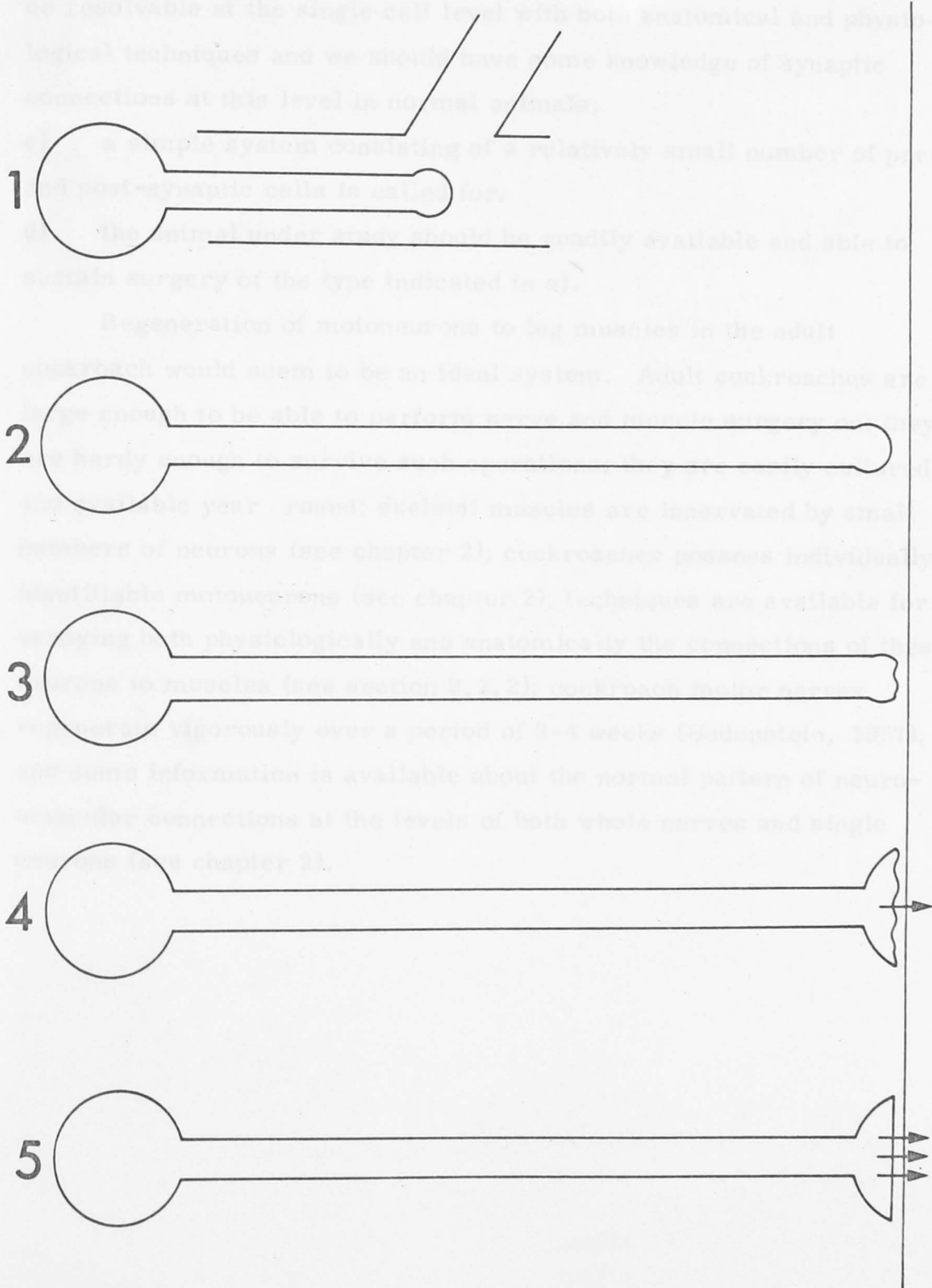
A suitable experimental system in which to approach these problems must have the following attributes:

- a) it should be possible to perform operations such as nerve crushes, muscle excision, limb transplantations, etc;
- b) the connections of neurons to their post-synaptic targets should

Fig. 1

Stages in Neuronal Development

1. Axon Outgrowth: a neuron is at this stage if it has sent out an axon (determined from anatomical evidence - cobalt backfilling, orthograde dye filling) but this axon has not yet arrived at the target cell in question (determined from anatomical evidence).
2. Neuron at Site of Target Cell: a neuron is at this stage if its axon can be detected at the site of the target cell (determined from anatomical evidence) but when no signs of synapse formation are evident (determined from anatomical evidence).
3. Non-Functional Synaptic Stage: a neuron is at this stage if morphological signs of synapse formation can be detected but no signs of physiological activity are evident.
4. Functional, Unstable Synaptic Stage: a neuron is at this stage if physiological signs of activity can be detected in the target cell upon stimulation of the neuron. At this stage the synapse is unstable in the sense that, if it remains at stage 4, it will eventually regress to the non-functional stage (or earlier stages).
5. Functional, Stable Synaptic Stage: a neuron is at this stage if physiological signs of activity can be detected in the target cell upon stimulation of the neuron at all indefinite times after innervation.



be resolvable at the single cell level with both anatomical and physiological techniques and we should have some knowledge of synaptic connections at this level in normal animals;

- c) a simple system consisting of a relatively small number of pre- and post-synaptic cells is called for;
- d) the animal under study should be readily available and able to sustain surgery of the type indicated in a).

Regeneration of motoneurons to leg muscles in the adult cockroach would seem to be an ideal system. Adult cockroaches are large enough to be able to perform nerve and muscle surgery on; they are hardy enough to survive such operations; they are easily cultured and available year round; skeletal muscles are innervated by small numbers of neurons (see chapter 2); cockroaches possess individually identifiable motoneurons (see chapter 2); techniques are available for assaying both physiologically and anatomically the connections of these neurons to muscles (see section 2.2.2); cockroach motor nerves regenerate vigorously over a period of 3-4 weeks (Bodenstein, 1957); and some information is available about the normal pattern of neuromuscular connections at the levels of both whole nerves and single neurons (see chapter 2).

2.1 INTRODUCTION

A detailed knowledge of gross neuroanatomy and neuromuscular connections at the individual neuron level in normal adult cockroaches is an essential prerequisite to any study of the possibility of reinnervation in the motor system. In the case of the cockroach, *Periplaneta americana*, much of this information is already available. Oatridge and Tiedel (1968) have described the gross morphology of the nervous system: the ventral nerve cord consists of three (sub- and meta-) thoracic ganglia and six abdominal ganglia. The bilaterally symmetrical thoracic ganglia, which are joined by paired longitudinal connectives, each give rise to eight nerves, four of which supply leg muscles. The pattern of branching of the peripheral nerves and their terminations have been worked out in detail by Pigg and Cook (1968).

CHAPTER 2

NEUROANATOMY AND NEUROMUSCULAR CONNECTIONS IN NORMAL ADULT COCKROACHES

The pattern of motor nerve cell bodies on the surface of the cockroach meso- and meta-thoracic ganglia have been determined by dissection from serial sections (Pigg and Jackson, 1967; Young, 1971). Individual cell bodies have been numbered on the basis of their shape, shape and relative position. Fig. 2 shows such a map prepared by Young (1971) for the mesothoracic ganglion. Homologous cell bodies can be identified in the metathoracic ganglion.

Several workers have addressed the question of which of the motor neurons send axons out which peripheral nerves. Cohen and Jackson (1967) using the retrograde degeneration technique have identified and numbered the axons exiting from nerves 2, 3, 4, 5, 6 of the metathoracic ganglion. The retrograde degeneration technique has been used to determine the axonal contribution of motor neurons to peripheral nerves of prothoracic (Rex, 1970), mesothoracic (Wiggans, 1974), and metathoracic (Pearson and Fourtner, 1973; Lee and Anthony, 1971) ganglia. In most of the latter studies, however, axons contributing to a given nerve were identified as a group rather

2.1 INTRODUCTION

A detailed knowledge of gross neuroanatomy and neuromuscular connections at the individual neuron level in normal adult animals is an essential prerequisite to any study of the specificity of reinnervation in the motor system. In the case of the cockroach, Periplaneta americana, much of this information is already available. Guthrie and Tindall (1968) have described the gross morphology of the nervous system: the ventral nerve cord consists of three (pro-meso-and meta-) thoracic ganglia and six abdominal ganglia. The bilaterally symmetrical thoracic ganglia, which are joined by paired longitudinal connectives, each give rise to eight nerves on each side, four of which supply leg muscles. The pattern of branching of the peripheral nerves and their terminations have been worked out in detail by Pipa and Cook (1959) and Nijenhuis and Dresden (1955). The thoracic and leg musculature have been described and numbered by Carbonell (1947) and Dresden and Nijenhuis (1953).

The patterns of motoneuron cell bodies on the surfaces of the cockroach meso- and meta-thoracic ganglia have been determined by reconstruction from serial sections (Cohen and Jacklett, 1967; Young, 1969). Individual cell bodies have been numbered on the basis of their sizes, shapes and relative positions: fig. 2 shows such a map prepared by Young (1973) for the mesothoracic ganglion. Homologous neurons can be identified in the metathoracic ganglion.

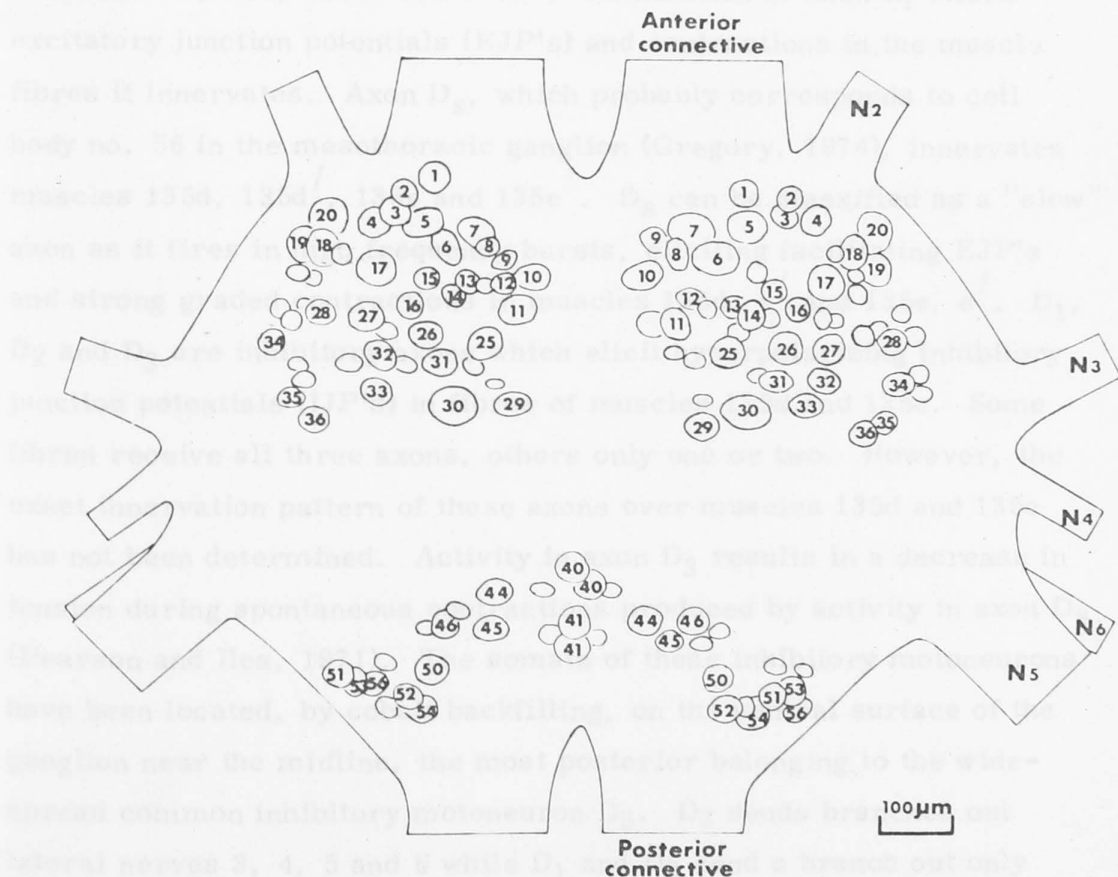
Several workers have addressed the question of which of the motoneuron somata send axons out which peripheral nerves. Cohen and Jacklett (1967) using the retrograde degeneration technique have identified and numbered the somata sending axons out nerves 2, 3, 4, 5, 6 in the metathoracic ganglion. Retrograde dye-filling techniques have been used to determine the axonal contribution of motoneuron somata to peripheral nerves of prothoracic (Iles, 1976), mesothoracic (Gregory, 1974), and metathoracic (Pearson and Fournier, 1973; Iles and Mulloney, 1971) ganglia. In most of the latter studies, however, neurons contributing to a given nerve were identified as a group rather

Fig. 2

Map of the cockroach mesothoracic ganglion showing the distribution of neuron cell bodies over 20 μ m in diameter. Ventral view (from Young, 1973).

than by their individual cell map numbers.

The pattern of connections at the single neuron level is known in detail for one system, the nerve 3 coxal depressor muscle group (Pearson and Hux, 1970, 1971; Hux and Pearson, 1973). The latter consists of six muscles, 135d, e, f, g, h, i, 135 and 137 which are innervated by branch 5r1 of nerve 3 (Fig. 3). Branch 5r1 contains five axons: D₁, D₂, D₃, D₄ and D₅. Axon D₁ corresponds to motoneuron no. 30 in the mesothoracic ganglion (Hux, 1973) and innervates muscles 137, 135, 135d and 135e. Stimulation of axon D₁ elicits excitatory junction potentials (EJPs) which are blocked by the muscle fibres it innervates. Axon D₂, which probably corresponds to cell body no. 56 in the mesothoracic ganglion (Gregory, 1974), innervates muscles 135d, 135e, 135f and 135g. Stimulation of axon D₂ elicits EJPs which are blocked by the muscle fibres it innervates. Axon D₃ innervates muscles 135f and 135g. Stimulation of axon D₃ elicits EJPs which are blocked by the muscle fibres it innervates. Axon D₄ innervates muscles 135g and 135h. Stimulation of axon D₄ elicits EJPs which are blocked by the muscle fibres it innervates. Axon D₅ innervates muscles 135h and 135i. Stimulation of axon D₅ elicits EJPs which are blocked by the muscle fibres it innervates.



lateral nerves 3, 4, 5 and 6 while D₁ and D₂ branch out only to nerve 3 (Pearson and Fourtner, 1973). The somata of these neurons have not been definitely numbered although Gregory (1974) suggests that D₁, D₂ and D₃ correspond to cells 40, 41 and 42 respectively in Young's (1973) cell map. The innervation of the nerve 3 coxal depressors is summarized in Fig. 4. Three other coxal depressor muscles act in parallel with the nerve 3 coxal depressor group described above; these are muscles 135a, b and c, innervated by

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The pattern of connections at the single neuron level is known in detail for one system, the nerve 5 coxal depressor muscle group (Pearson and Iles, 1970, 1971; Iles and Pearson, 1971). The latter consists of six muscles; 135d, d', e, e', 136 and 137 which are innervated by branch 5rl of nerve 5 (fig. 3). Branch 5rl contains five axons: D_f, D_s, D₁, D₂ and D₃. Axon D_f corresponds to motoneuron no. 30 in the mesothoracic ganglion (Iles, 1972) and innervates muscles 137, 136, 135d' and 135e'. Stimulation of axon D_f elicits excitatory junction potentials (EJP's) and contractions in the muscle fibres it innervates. Axon D_s, which probably corresponds to cell body no. 56 in the mesothoracic ganglion (Gregory, 1974), innervates muscles 135d, 135d', 135e and 135e'. D_s can be classified as a "slow" axon as it fires in high frequency bursts, eliciting facilitating EJP's and strong graded contractions in muscles 135d, d' and 135e, e'. D₁, D₂ and D₃ are inhibitory axons which elicit hyperpolarizing inhibitory junction potentials (IJP's) in fibres of muscles 135d and 135e. Some fibres receive all three axons, others only one or two. However, the exact innervation pattern of these axons over muscles 135d and 135e has not been determined. Activity in axon D₃ results in a decrease in tension during spontaneous contractions produced by activity in axon D_s (Pearson and Iles, 1971). The somata of these inhibitory motoneurons have been located, by cobalt backfilling, on the ventral surface of the ganglion near the midline, the most posterior belonging to the wide-spread common inhibitory motoneuron D₃. D₃ sends branches out lateral nerves 3, 4, 5 and 6 while D₁ and D₂ send a branch out only nerve 5 (Pearson and Fournier, 1973). The somata of these neurons have not been definitely numbered although Gregory (1974) suggests that D₁, D₂ and D₃ correspond to cells 40, 44 and 41 respectively in Young's (1973) cell map. The innervation of the nerve 5 coxal depressors is summarised in fig. 4. Three other coxal depressor muscles act in parallel with the nerve 5 coxal depressor group described above: these are muscles 135a, b and c, innervated by

Fig. 3

Neuromuscular Anatomy of Mesothoracic Coxa
in the Adult Cockroach

- A. Ventral view of coxa showing depressor muscle 137 and levator muscles 138 and 139c. The latter muscles are innervated by branches of nerve 3B.
- B. Muscle 137 removed to show depressor muscles 135d, d' and 135e, e'.
- C. Muscles 135d, d', e, e' removed to show depressor muscles 135a, b, c and 136. 135a, b, c originate from different points in the thorax and are innervated by branches from nerve 4. Sub-branches of branch 5rl of nerve 5 innervate muscles 136, 135d, d', 135e, e' and 137.

The lines marked CN3b, CN5, CM indicate the level at which nerves 3B, 5 and the coxal depressor muscles, respectively, were cut in the muscle excision operation (see chapter 3).

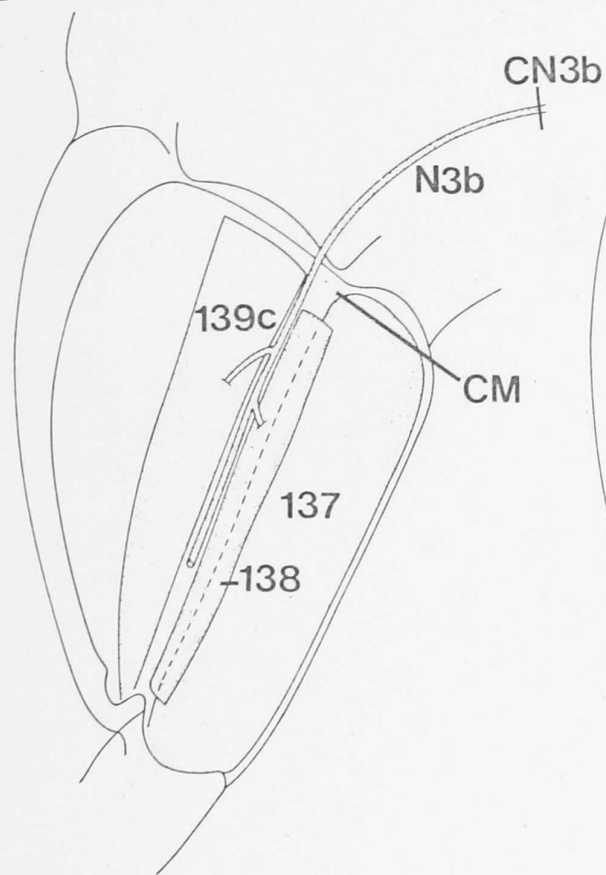
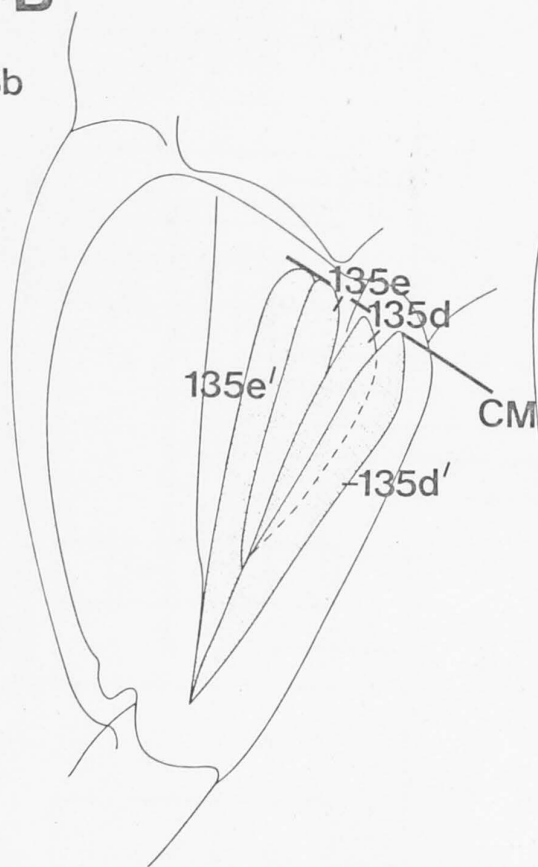
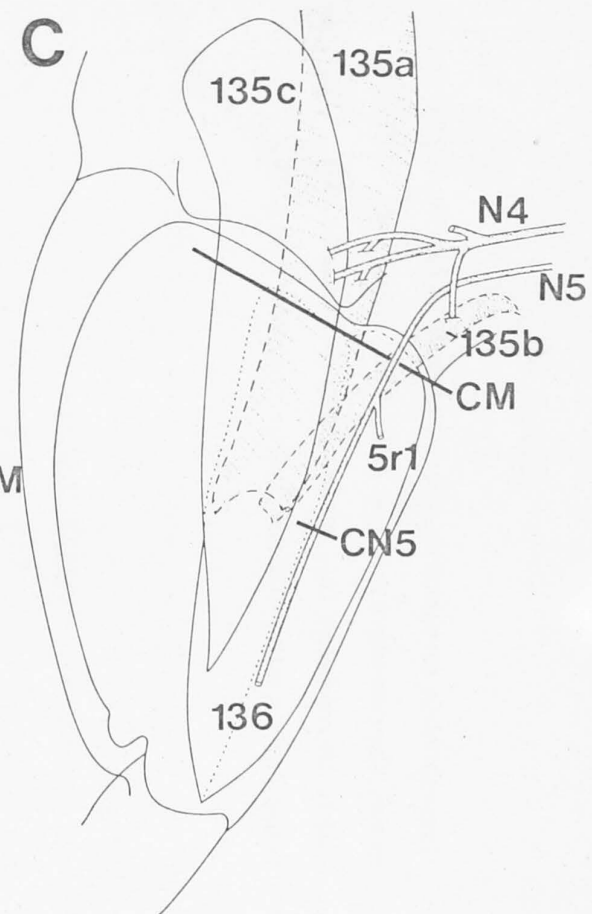
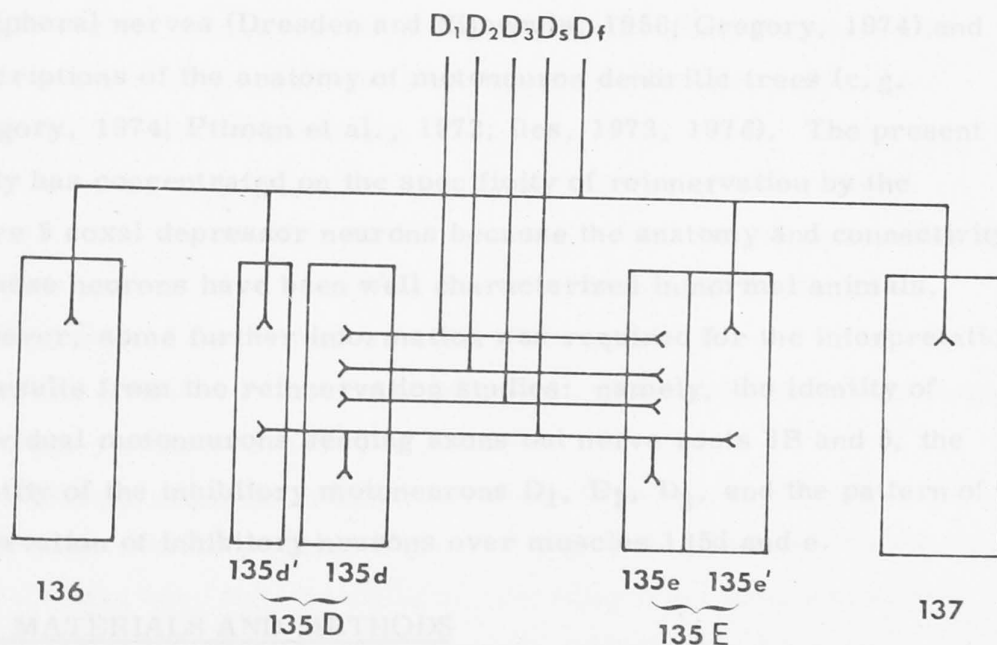
A**B****C**

Fig. 4

Distribution of the five motor axons in nerve 5r1 to the mesothoracic coxal depressor muscles 136, 137, 135d, d', 135e, e'. D_f, fast coxal depressor; D_s, slow coxal depressor; D₁, D₂, D₃, common inhibitory motoneurons (from Pearson and Iles, 1971).

horacic
fast
amon



2.2.1 REARING OF COCKROACHES

Cockroaches were obtained from a colony housed on cardboard in a large (four-gallon) bin. They were given a continuous supply of rat pellets and water and maintained at a temperature of 16 to 20°C. Vigorous adult male cockroaches were used exclusively in all experiments.

2.2.2 ELECTROPHYSIOLOGICAL TECHNIQUES

2.2.2.1 Setting up. The cockroach was anaesthetized with CO₂ and placed

branches of nerve 4 (fig. 3).

Aside from the coxal depressor neurons, the peripheral connections of anatomically identified cockroach motoneurons are known in only a few other cases. Young (1972) has shown that mesothoracic motoneuron no. 29 innervates muscles 118 and 126 while cell no. 18 innervates coxal depressor muscle 135a.

Metathoracic motoneurons, anatomically homologous to these mesothoracic cells, innervate homologous metathoracic muscles.

Other useful background information for regeneration studies includes estimates of numbers of motor and sensory fibres in peripheral nerves (Dresden and Nijenhuis, 1958; Gregory, 1974) and descriptions of the anatomy of motoneuron dendritic trees (e.g. Gregory, 1974; Pitman et al., 1972; Iles, 1972, 1976). The present study has concentrated on the specificity of reinnervation by the nerve 5 coxal depressor neurons because the anatomy and connectivity of these neurons have been well characterized in normal animals. However, some further information was required for the interpretation of results from the reinnervation studies: namely, the identity of individual motoneurons sending axons out nerve roots 3B and 5, the identity of the inhibitory motoneurons D₁, D₂, D₃, and the pattern of innervation of inhibitory neurons over muscles 135d and e.

2.2 MATERIALS AND METHODS

2.2.1 REARING OF COCKROACHES

Cockroaches were obtained from a colony housed on cardboard egg cartons in a large fibreglass bin. They were given a continuous supply of rat pellets and water and maintained at a temperature of 18 to 26°C. Vigorous adult male cockroaches were used exclusively in all experiments.

2.2.2 ELECTROPHYSIOLOGICAL TECHNIQUES

A. Setting up. The cockroach was anaesthetized with CO₂ and placed

ventral side up on a wax-bottomed preparation tray. The legs were stretched out and immobilized with plasticine and insect wax. A dam of petroleum jelly (vaseline) was constructed to allow a pool of saline to be retained over the ganglion.

The cuticle covering the ventral surface of the coxa was removed, taking care not to damage muscle fibres. The basisternum, together with underlying fat deposits and tracheae were dissected away to expose the mesothoracic ganglion. A small spoon, fashioned from the tip of a No. 18 syringe needle, and covered with soft plastic, was slipped between the anterior connectives and positioned under the ganglion. Stainless steel insect pins were driven into the plastic covering the spoon adjacent to the anterior and posterior connectives to reduce movements of the ganglion (fig. 5).

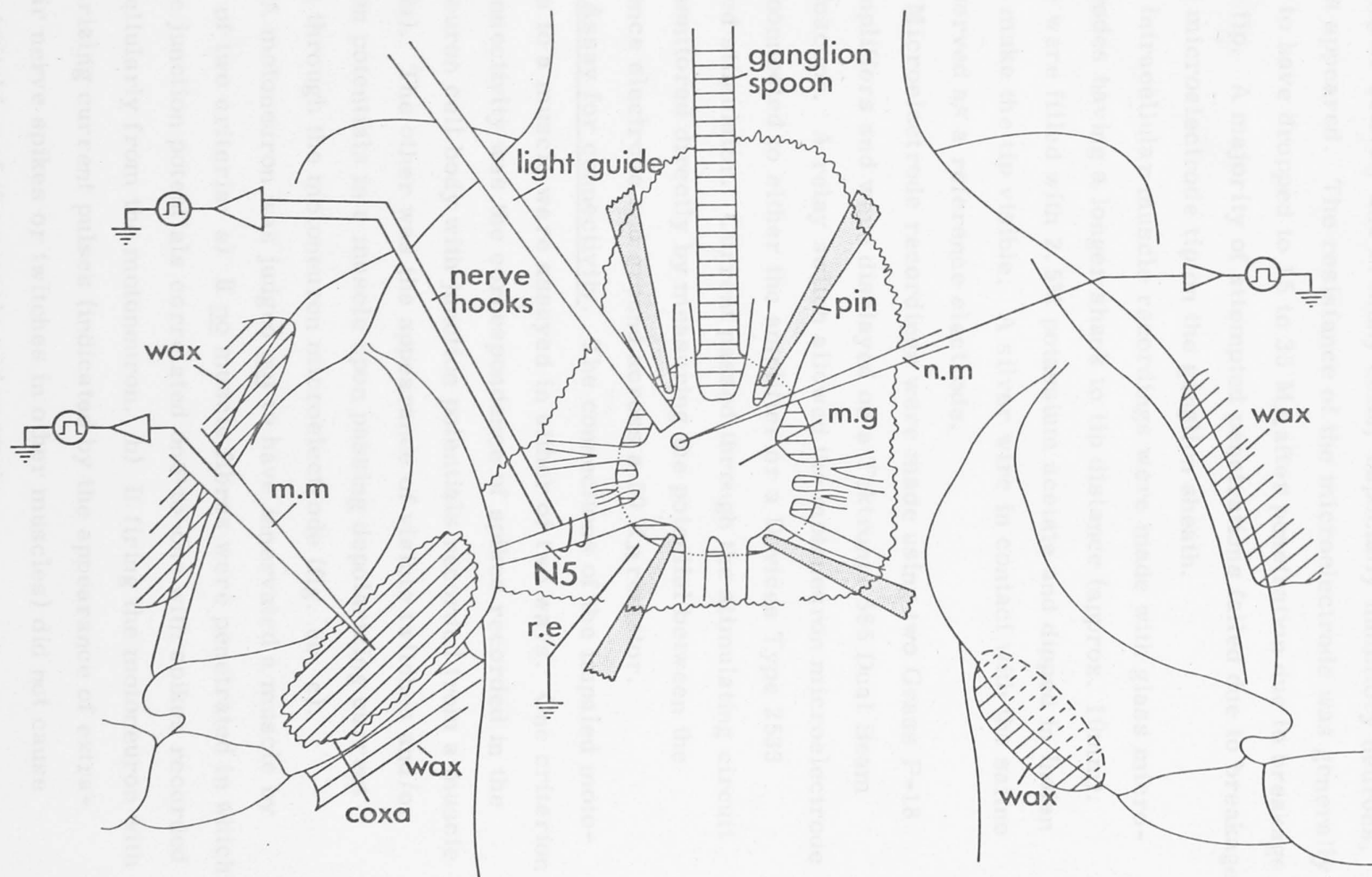
A light guide, made by drawing out a piece of glass rod, provided side illumination of the ganglion. With careful positioning of this light source it was possible to visualise the cell bodies of motoneurons on the ventral surface of the ganglion.

The ganglion was covered with the cockroach saline of Pearson, Wong and Fourtner (1976).

B. Intracellular Recording. Microelectrodes, pulled from fibre-filled glass capillary tubing (O.D. 1.0 mm, I.D. 0.5 mm), on a Kopf puller, were used for intracellular recording from motoneuron cell bodies. Microelectrodes were approximately 6.5mm from tip to shank and showed an even taper to the tip. They were filled with 4% Procion brown (I.C.I.) through a No. 30 syringe needle. The resistance of such microelectrodes ranged from 60 to 160 M Ω . The microelectrode was slowly lowered until its tip lightly touched the sheath of the ganglion. This was indicated by a positive shift in D.C. level. The electrode holder was then tapped gently while the electrode was lowered still further. Eventually a point was reached where lowering the electrode gave no further shift in D.C. level. At this point the electrode holder was given a sharp tap or several light taps. In successful preparations tapping was followed by a sudden negative

Fig. 5

Ventral view of cockroach as set up for electrophysiological recording. m.g., mesothoracic ganglion; r.e., reference electrode; n.m., microelectrode recording from motoneuron soma; m.m., microelectrode recording from muscle fibre; N5, nerve 5.



D.C. shift of between 30 and 70 mV as the microelectrode penetrated the motoneuron cell body. At the same time the noise level rose due to synaptic activity and in many cells, especially inhibitory neurons, spikes appeared. The resistance of the microelectrode was generally found to have dropped to 15 to 35 M Ω after penetration due to breakage of the tip. A majority of attempted penetrations failed due to breakage of the microelectrode tip on the ganglion sheath.

Intracellular muscle recordings were made with glass microelectrodes having a longer shank to tip distance (approx. 10mm). These were filled with 2.5M potassium acetate and dipped in Indian Ink to make the tip visible. A silver wire in contact with the saline dam served as a reference electrode.

Microelectrode recordings were made using two Grass P-18 Preamplifiers and were displayed on a Tektronix 565 Dual Beam Oscilloscope. A relay switch allowed the motoneuron microelectrode to be connected to either the amplifier or a Devices Type 2533 isolated stimulator. Current passed through the stimulating circuit was monitored directly by measuring the potential between the reference electrode and ground across a 50 K Ω resistor.

C. Assay for connectivity. The connections of the impaled motoneuron to a muscle were assayed in either of two ways. One criterion for connectivity was the correspondence of spikes recorded in the motoneuron cell body with junction potentials recorded from a muscle (fig. 6a). The other was the appearance of visible twitches and/or junction potentials in a muscle upon passing depolarizing current pulses through the motoneuron microelectrode (fig. 6b, c).

A motoneuron was judged not to have innervated a muscle by either of two criteria: a) If no muscle fibres were penetrated in which muscle junction potentials correlated one-to-one with spikes recorded intracellularly from the motoneuron. b) If firing the motoneuron with depolarizing current pulses (indicated by the appearance of extracellular nerve spikes or twitches in other muscles) did not cause visible twitching of the muscle under study.

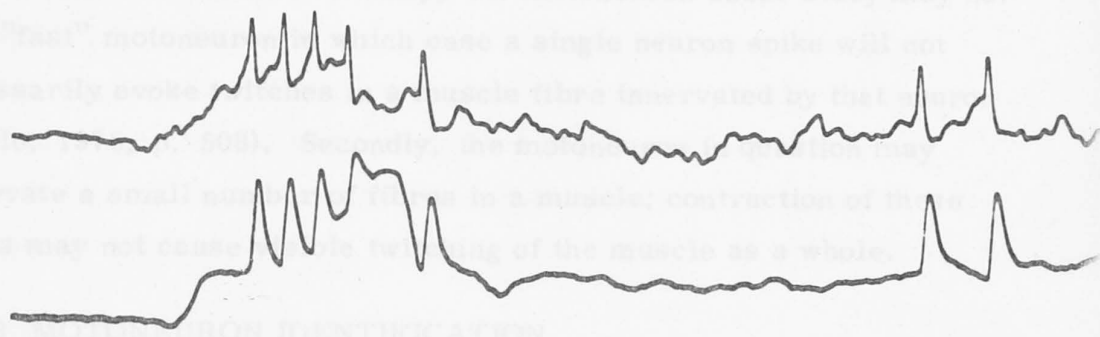
Fig. 6

Assays for Connectivity

- A. Intracellular recordings from motoneuron no. 30 (top trace) and muscle 139c (bottom trace). Note correspondence of muscle junction potentials with motoneuron spikes. Vertical calibration bar 5 mV. Horizontal bar 25 msec.
- B, C. Excitatory junction potentials recorded from muscle 139c (top trace) upon passage of depolarizing current pulses through microelectrode in motoneuron no. 30 (bottom trace). Vertical calibration 5 mV (top trace), 10 μ A (bottom trace). Horizontal bar fig. B 30 msec fig. C 50 msec.

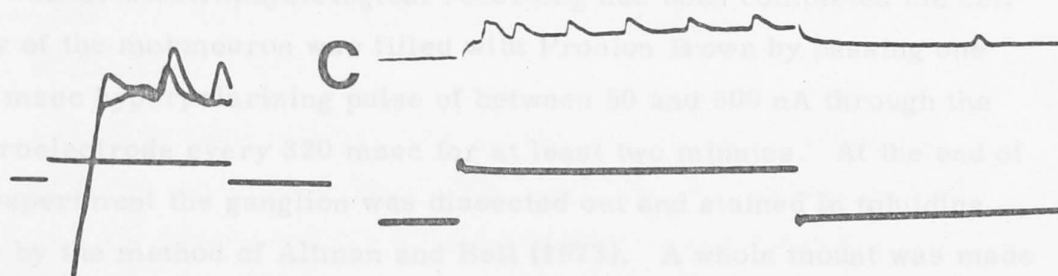
A

L

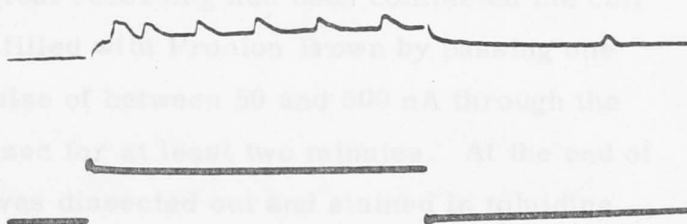


B

L



C



Both of these assays are open to criticism: the first on the grounds that the microelectrode may penetrate some fibers of the muscle under study but not those penetrated. Alwood and Hoyle (1985) have shown that some fibers in crayfish muscle are difficult to penetrate with microelectrodes. It is not possible to counter this criticism unless every fiber in a muscle can be recorded from, a condition which was not met in the present study. The second assay may be invalid for at least two reasons:—firstly, the motor neuron under study may not be a "fast" motor neuron, in which case a single neuron spike will not necessarily produce a noticeable contraction of the muscle fibers (Hoyle 1980). Secondly, the motor neuron may be a slow motor neuron, in which case a single neuron spike will not produce a noticeable contraction of the muscle fibers (Hoyle 1980).

3.3.3.3. NOTATION

After electrophysiological recording had been completed the cell body of the motor neuron was removed from the muscle. The body of the motor neuron was removed by cutting the nerve between 100 and 200 microns from the muscle. The nerve was then cut at the end of the muscle. The nerve was then cut at the end of the muscle. The nerve was then cut at the end of the muscle.

3.3.4. DYE DIFFUSION TECHNIQUE

The identity of motor neurons exiting across gut particular nerve branches was established by double labelling techniques. The ganglion, together with a length of the nerve under study was dissected from the animal and placed in saline in one of a diffusion chamber consisting of two shallow wells drilled close together in a sheet of perspex, so that they are separated by a thin wall. The nerve was drawn across a reaction bridge in a narrow slot cut in the wall between the chambers,

Both of these assays are open to criticism: the first on the grounds that the motoneuron may innervate some fibres of the muscle under study but not those penetrated. Atwood and Hoyle (1965) have shown that some fibres in crayfish muscles are difficult to penetrate with microelectrodes. It is not possible to counter this criticism unless every fibre in a muscle can be recorded from, a condition which was not met in the present study. The second assay may be invalid for at least two reasons. Firstly, the motoneuron under study may not be a "fast" motoneuron in which case a single neuron spike will not necessarily evoke twitches in a muscle fibre innervated by that neuron (Hoyle, 1975, p. 508). Secondly, the motoneuron in question may innervate a small number of fibres in a muscle; contraction of these fibres may not cause visible twitching of the muscle as a whole.

2.2.3 MOTONEURON IDENTIFICATION

After electrophysiological recording had been completed the cell body of the motoneuron was filled with Procion Brown by passing one 100 msec hyperpolarizing pulse of between 50 and 500 nA through the microelectrode every 320 msec for at least two minutes. At the end of an experiment the ganglion was dissected out and stained in toluidine blue by the method of Altman and Bell (1973). A whole mount was made of the ganglion and a diagram of motoneuron cell body positions made using a Nikon microscope and Wild drawing tube. The marked cell bodies were numbered by reference to Young's (1973) cell map of the mesothoracic ganglion.

2.2.4 DYE DIFFUSION TECHNIQUE

The identity of motoneurons sending axons out particular nerve branches was established by cobalt backfilling techniques. The ganglion, together with a length of the nerve under study, was dissected from the animal and placed in saline in one well of a diffusion chamber consisting of two shallow wells drilled close together in a sheet of perspex, so that they are separated by a thin wall. The nerve was drawn across a vaseline bridge in a narrow slot cut in the wall between the chambers,

placed in a solution of 5% cobalt chloride in the opposite chamber and recut. The chamber was placed in a moist atmosphere overnight allowing cobalt chloride to diffuse up the cut axons. The ganglion was then removed from the well, washed in cockroach saline and bathed in 10% ammonium sulphide solution for 5 minutes. After a 5 minute wash in saline the ganglion was fixed in Bodian's No. 2 fixative, dehydrated through an ethanol series, cleared in xylene and mounted in Permount. Filled neurons were drawn using a Nikon microscope and Wild drawing tube. The ganglion was returned to xylene, rehydrated and stained in Toluidine blue to enable a map of motoneuron cell bodies to be made as described on page 26. The filled cells were then numbered by comparison with the mesothoracic cell map of Young (1973).

2.3 RESULTS

2.3.1 IDENTIFICATION OF SOMATA OF NEURONS WITH AXONS IN NERVES 5 AND 3B

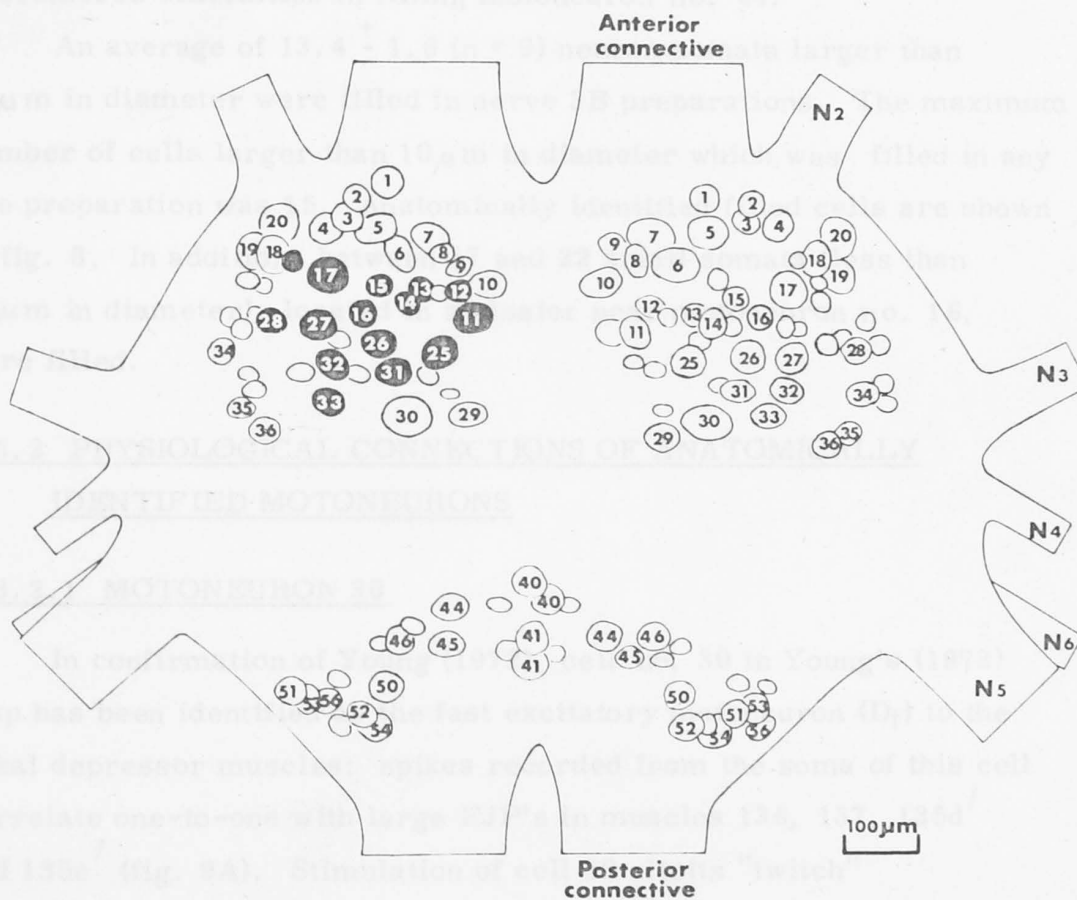
Cobalt backfill preparations reveal the location of somata of neurons which send axons out nerves 5 and 3B. An average of 21.8 ± 2.3 ($n = 10$) neuron somata were filled when nerve 5 was cut just distal to nerve 5rl. The maximum number of cells which were filled in any one preparation was 25. Where possible the neurons were numbered by comparison with Young's (1973) cell map. Fig. 7 shows motoneurons which filled in at least one preparation. The axons of two or three of the motoneurons filled through nerve 5 exit the ganglion via nerve 4 and enter nerve 5 through the small commissure linking these nerves (see fig. 16). These somata are in a group removed in an anterior, lateral direction from the main group of nerve 5 cell bodies.

Cobalt backfills on mesothoracic nerve 5rl consistently fill four cell bodies, namely nos. 30, 40, 44 and D₅, as described previously in the metathoracic ganglion (Pearson and Fournier, 1973). Moto-

Fig. 7

Cell map of mesothoracic ganglion (from Young, 1973) showing motoneurons stained when nerve 5 is backfilled with cobalt chloride (stained cells shown in black).

neuron No. 41 is filled in only approximately 10 to 20% of preparations. This result is believed to be an artifact of the cobalt technique as a small axon, probably belonging to motoneuron no. 41 (see Pearson and Fourtner, 1973) could be seen in the commissure between nerves 4 and 5 in almost all preparations. Denburg et al. (1977) have also encountered difficulties in filling motoneuron no. 41.



neuron No. 41 is filled in only approximately 20 to 30% of preparations. This result is believed to be an artefact of the cobalt technique as a small axon, probably belonging to motoneuron no. 44 (see Pearson and Fournier, 1973) could be seen in the commissure between nerves 4 and 5 in almost all preparations. Denburg et al. (1977) have also encountered difficulties in filling motoneuron no. 44.

An average of 13.4 ± 1.6 ($n = 9$) neuron somata larger than $10\mu\text{m}$ in diameter were filled in nerve 3B preparations. The maximum number of cells larger than $10\mu\text{m}$ in diameter which was filled in any one preparation was 15. Anatomically identified filled cells are shown in fig. 8. In addition, between 17 and 22 small somata (less than $10\mu\text{m}$ in diameter), located in a cluster near motoneuron no. 16, were filled.

2.3.2 PHYSIOLOGICAL CONNECTIONS OF ANATOMICALLY IDENTIFIED MOTONEURONS

2.3.2.1 MOTONEURON 30

In confirmation of Young (1972), cell no. 30 in Young's (1973) map has been identified as the fast excitatory motoneuron (D_f) to the coxal depressor muscles: spikes recorded from the soma of this cell correlate one-to-one with large EJP's in muscles 136, 137, 135d' and 135e' (fig. 9A). Stimulation of cell 30 elicits "twitch" contractions in the latter muscles. Cell 30 innervates all fibres of muscles 135d', e', 136 and 137 which have been recorded from. No junction potentials, apart from those which correlate with spike activity in cell 30, have been recorded from muscles 136 and 137 in normal animals. An additional small EJP has been seen in all fibres of muscles 135d', e' recorded from. These small EJP's correlate one-to-one with large tonic spikes recorded extracellularly from nerve 5 (fig. 9B) and are presumed to result from activity in D_S (see section 4.2.2 for extracellular nerve 5 recording technique). This is the only large tonic unit which appears in extracellular nerve 5

Fig. 8

Cell map of mesothoracic ganglion (from Young, 1973) showing motoneurons stained when nerve 3B is backfilled with cobalt chloride (stained cells shown in black). Asterisk indicates position of cluster of small filled neurons (see text).

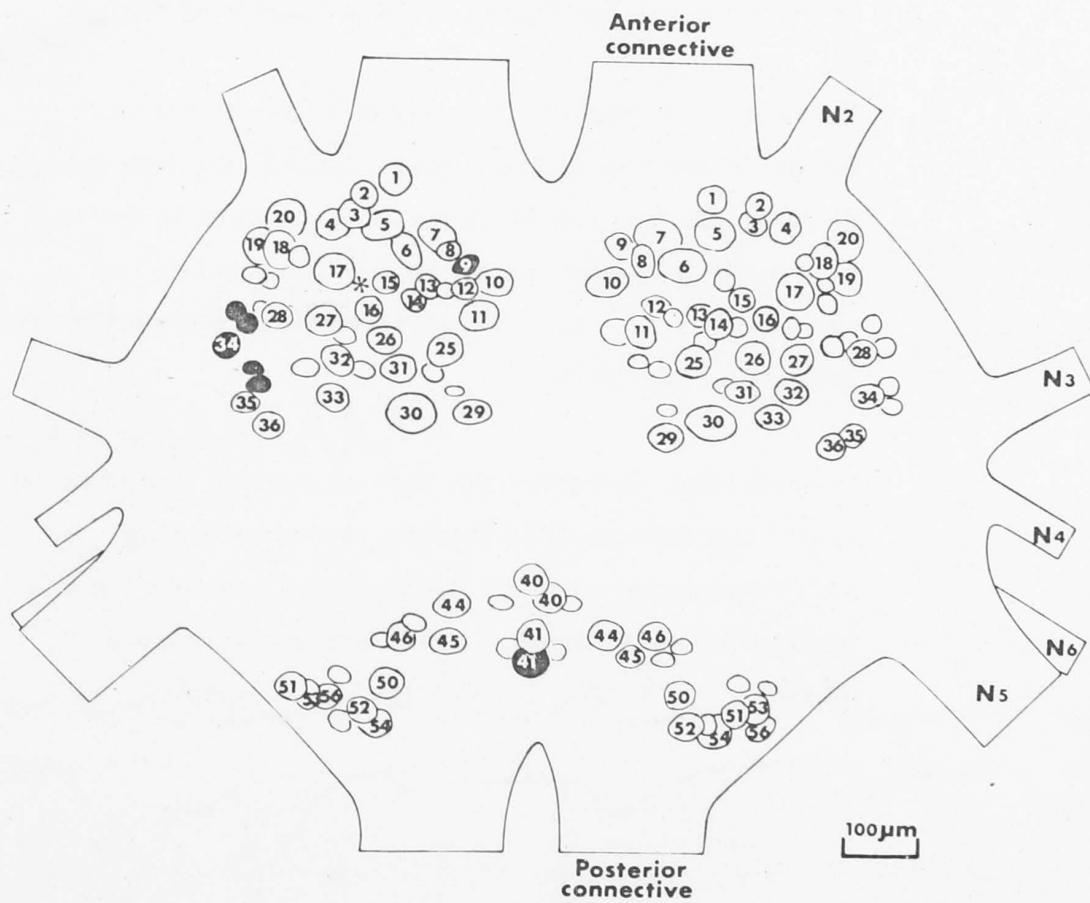
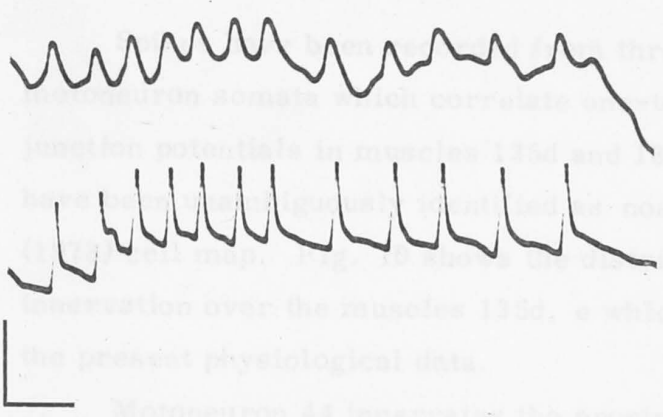


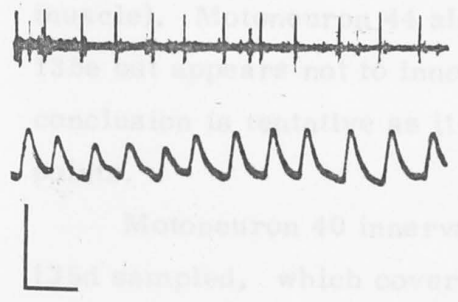
Fig. 9

- A. Intracellular records from motoneuron no. 30 soma (top trace) and muscle 137 (bottom trace) in normal adult cockroach, showing correspondence of spikes in motoneuron no. 30 with excitatory junction potentials in muscle 137. Vertical calibration bar 5 mV. Horizontal calibration bar 25 msec.
- B. Extracellular record from nerve 5 (top trace) and intracellular record from muscle 135d' (bottom trace), showing correspondence between D_S spikes in nerve 5 with excitatory junction potentials in muscle 135d'. Vertical bar top trace 250 μ V; bottom trace 5 mV. Horizontal bar 30 msec.
- C. Intracellular records from motoneuron no. 44 soma (top trace) and muscle 135d (bottom trace), showing correspondence between spikes in the motoneuron with hyperpolarizing junction potentials in the muscle. Vertical bar 5 mV. Horizontal bar 10 msec.

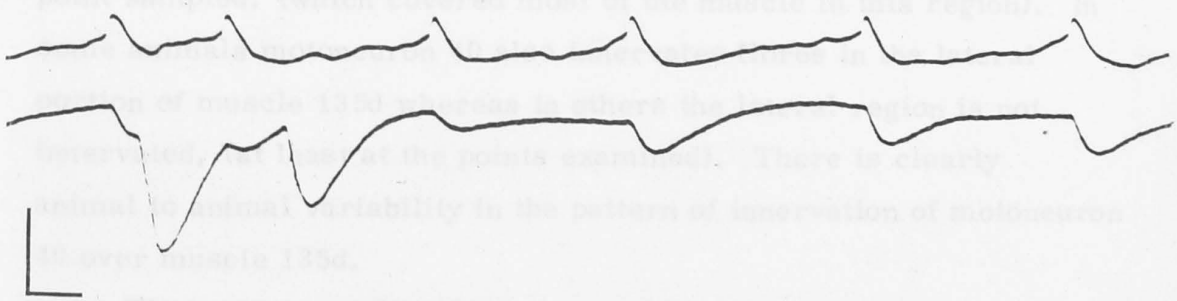
A



B



C



records. The soma of this motoneuron has not been identified by physiological methods.

2.3.3.2 INHIBITORY NEURONS

Three anatomically distinct motoneurons innervate muscle 135d in a one-to-one relationship. These are cells 40, 41 and 44 in Young's 1973 cell map. The motoneuron innervating muscle 135d at the most proximal point sampled but does not innervate muscle 135e at any of the points examined, (only a restricted part of the muscle sampled). The

Motoneuron 44 innervates the proximal part of muscle 135d at every point examined, which covered only the proximal part of the muscle. This motoneuron also innervates the proximal part of muscle 135e. This is based upon recordings at only three points. Motoneuron 40 innervates muscle 135d at every point on muscle 135d sampled, which covered only the distal part of this muscle.

Motoneuron 41 innervates the medial part of muscle 135d at every point sampled, which covered most of the muscle in this region. In muscle 135e it innervates the medial part of the muscle. There is clearly a high degree of variability in the pattern of innervation of motoneurons over muscle 135d.

The most posterior of the group of three posterior ventral motoneurons which innervate muscle 135d, which Pearson and Fourtner (1973) identify as the widespread common inhibitory motoneuron D₂ (Pearson and Ras, 1971) has been unambiguously identified as motoneuron 40, 41 in Young's 1973 cell map. This neuron innervates muscle 135d at the one point sampled but does not innervate muscle 135e at any of the points examined, (only a restricted part of the muscle sampled). The

records. The soma of this motoneuron has not been identified by physiological methods.

2.3.2.2 INHIBITORY NEURONS

Spikes have been recorded from three anatomically distinct motoneuron somata which correlate one-to-one with inhibitory junction potentials in muscles 135d and 135e (fig. 9C): these cells have been unambiguously identified as nos. 40, 41 and 44 in Young's (1973) cell map. Fig. 10 shows the distribution of inhibitory innervation over the muscles 135d, e which has been inferred from the present physiological data.

Motoneuron 44 innervates the proximal part of muscle 135d at every point examined, (which covered only the proximal part of the muscle). Motoneuron 44 also innervates the proximal part of muscle 135e but appears not to innervate the distal part of this muscle. This conclusion is tentative as it is based upon recordings at only three points.

Motoneuron 40 innervates muscle 135d at every point on muscle 135d sampled, which covered only the distal part of this muscle. The same neuron innervates the medial part of muscle 135e at every point sampled, (which covered most of the muscle in this region). In some animals motoneuron 40 also innervates fibres in the lateral portion of muscle 135d whereas in others the lateral region is not innervated, (at least at the points examined). There is clearly animal to animal variability in the pattern of innervation of motoneuron 40 over muscle 135d.

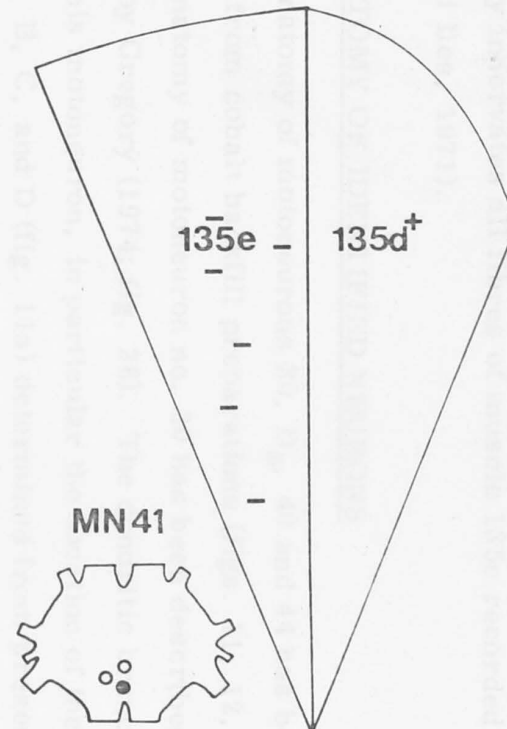
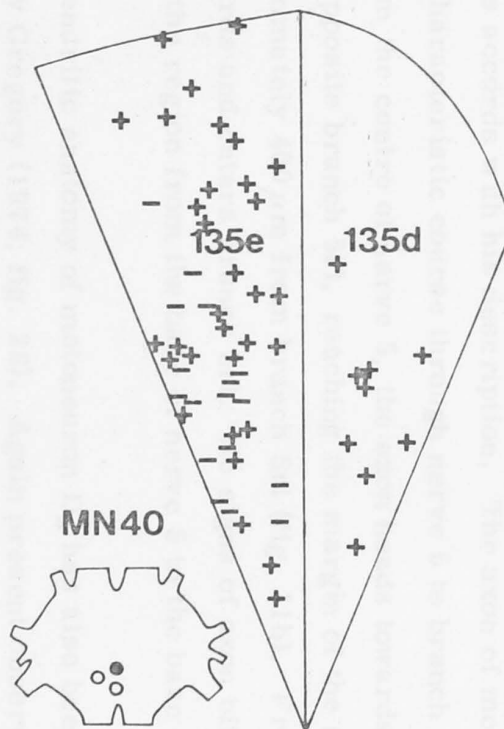
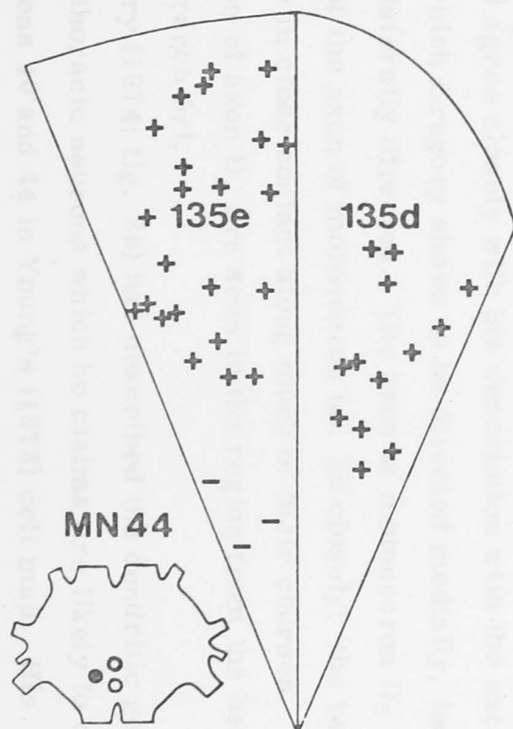
The most posterior of the group of three posterior ventral somata which fill through nerve 5r1, which Pearson and Fourtner (1973) identify as the widespread common inhibitory motoneuron D₃ (Pearson and Iles, 1971), has been unambiguously identified as motoneuron no. 41 in Young's 1973 cell map. This neuron innervates muscle 135d at the one point sampled but does not innervate muscle 135e at any of the points examined, (only a restricted part of the muscle sampled). The

Fig. 10

Distribution of Inhibitory Motoneuron
Innervation Over Muscles 135d, e.

- + = spike activity in the motoneuron in question correlates with junction potentials recorded from the muscle at this point.
- = no correlation between spike activity in the motoneuron and junction potentials recorded from the muscle at this point.

Pattern for motoneurons 44 and 40 based on observations from 8 animals and for motoneuron 41 from 3 animals.



latter result is at variance with the previous finding that motoneuron D₃ generally innervates all fibres of muscle 135e recorded from (Pearson and Iles, 1971).

2.3.3 ANATOMY OF IDENTIFIED NEURONS

The anatomy of motoneurons 30, D_S, 40 and 44 has been determined from cobalt backfill preparations (figs. 11, 12, 13, 14).

The anatomy of motoneuron no. 30 has been described previously by Gregory (1974; fig. 26). The dendritic branching pattern of this motoneuron, in particular the location of the major branches A, B, C, and D (fig. 11a) determined from present observations accords with his description. The axon of motoneuron 30 takes a characteristic course through nerve 5 to branch 5rl: starting from the centre of nerve 5, the axon heads towards the side of nerve 5 opposite branch 5rl, reaching the margin of the nerve at a point approximately 400 μ m from branch 5rl (fig. 11b). From here it curves towards and enters branch 5rl. No signs of axon bifurcation are seen in the region from the base of nerve 5 to the base of branch 5rl.

The dendritic anatomy of motoneuron D_S has also been described by Gregory (1974; fig. 26). Again present observations (see fig. 12) agree closely with his description with the exception that branch A, which Gregory shows to be directed medially, has been found to be laterally directed. The axon of motoneuron D_S follows the course of the axon of motoneuron no. 30 closely: the two axons appear to be in close contact along much of their courses. No signs of bifurcation of axon D_S are seen in the region from the base of nerve 5 to branch 5rl.

Gregory (1974; fig. 26) has described the dendritic geometry of two mesothoracic neurons which he claims are likely to correspond to motoneurons 40 and 44 in Young's (1973) cell map. His descriptions agree closely with present observations on the structure of motoneurons 40 and 44 (figs. 13, 14). Both of these neurons show

Fig. 11a

Dendritic anatomy of motoneuron 30 in normal animals.

N2, N3, N4, N5, peripheral nerve trunks 2, 3, 4 and 5.

AC, anterior connective; PC, posterior connective.

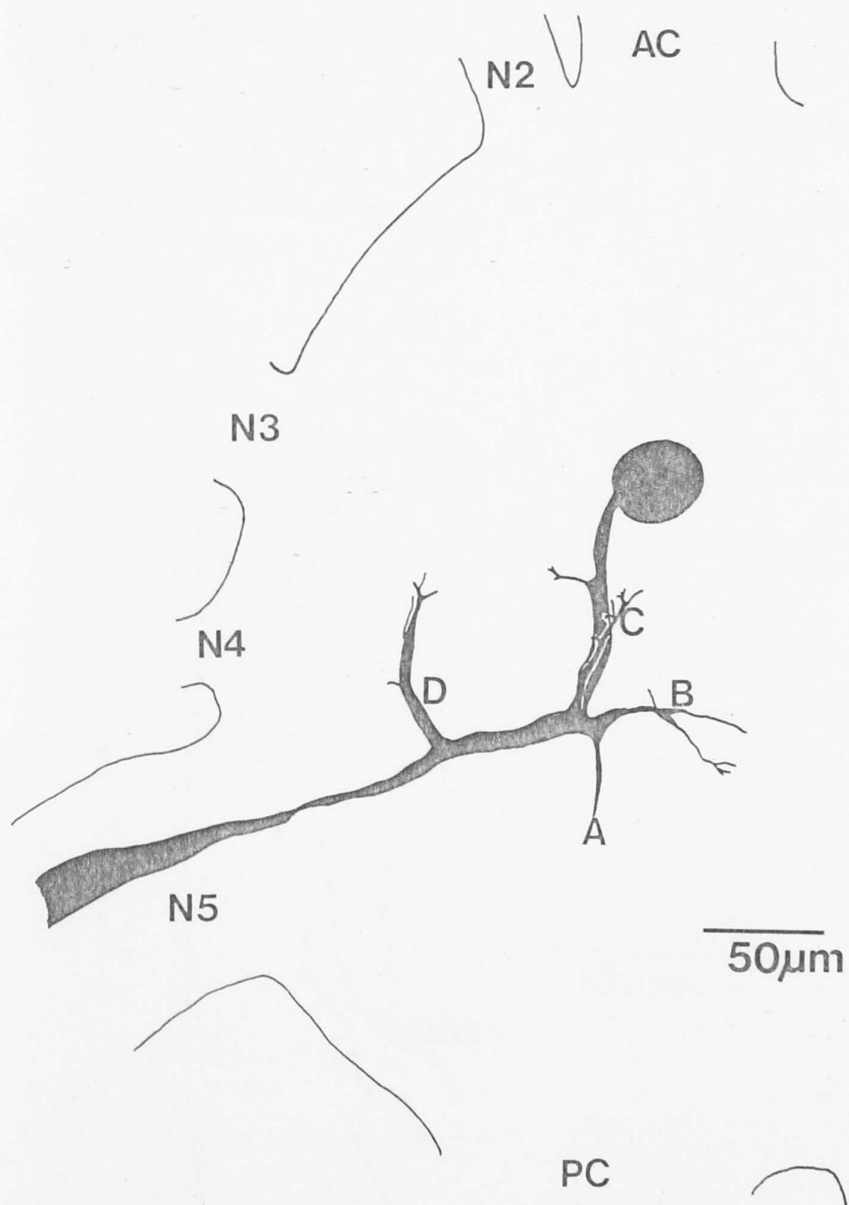


Fig. 11b

Axonal anatomy of motoneuron 30 in normal animals.

N5, base of nerve 5; N5rl, nerve 5rl, arrow points in anterior direction.

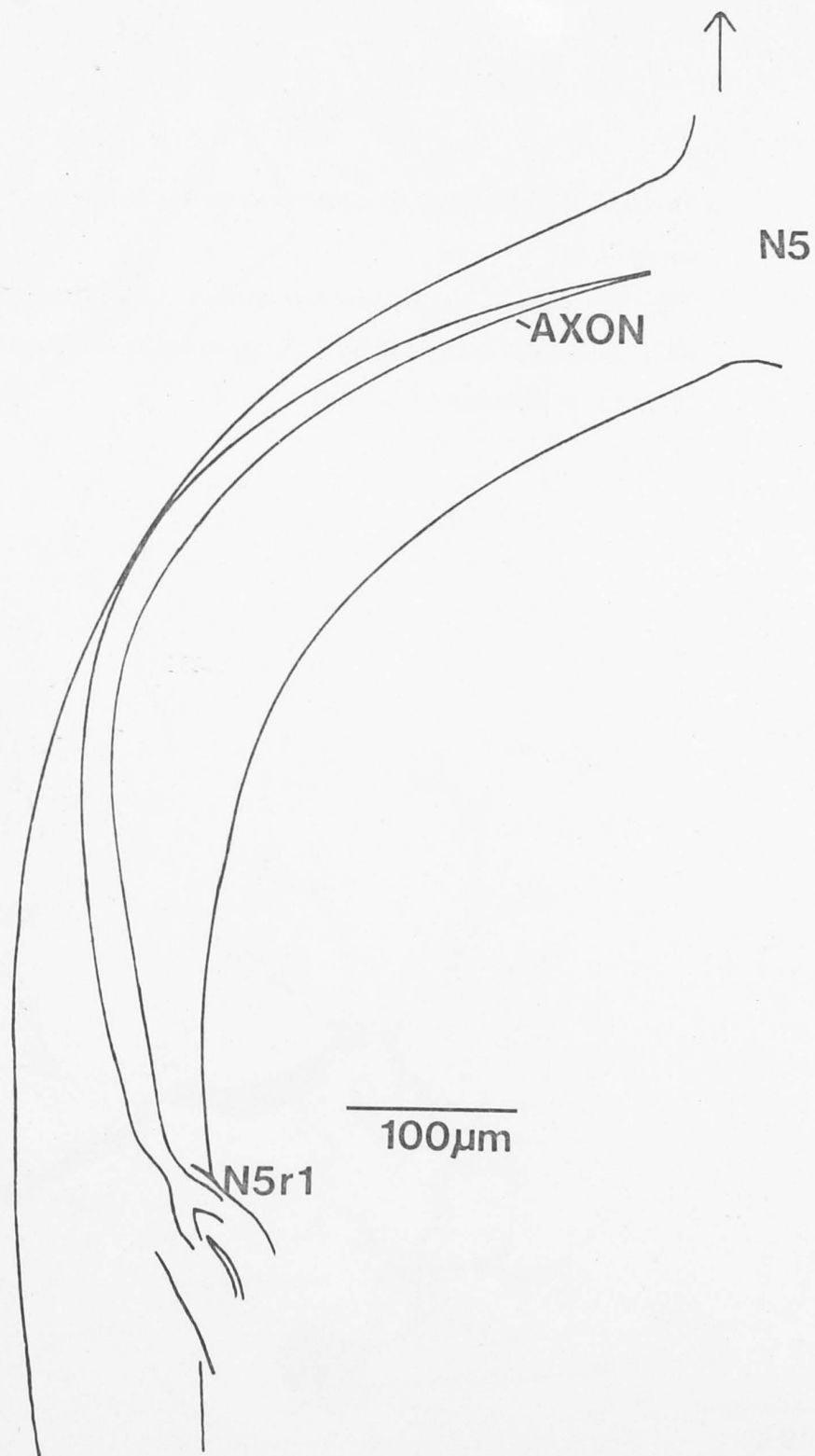


Fig. 12

Dendritic geometry of motoneuron D_S in normal animals.

N2, N3, N4, N5, peripheral trunks 2, 3, 4 and 5.

AC, anterior connective; PC, posterior connective.

Branch A discussed in text.

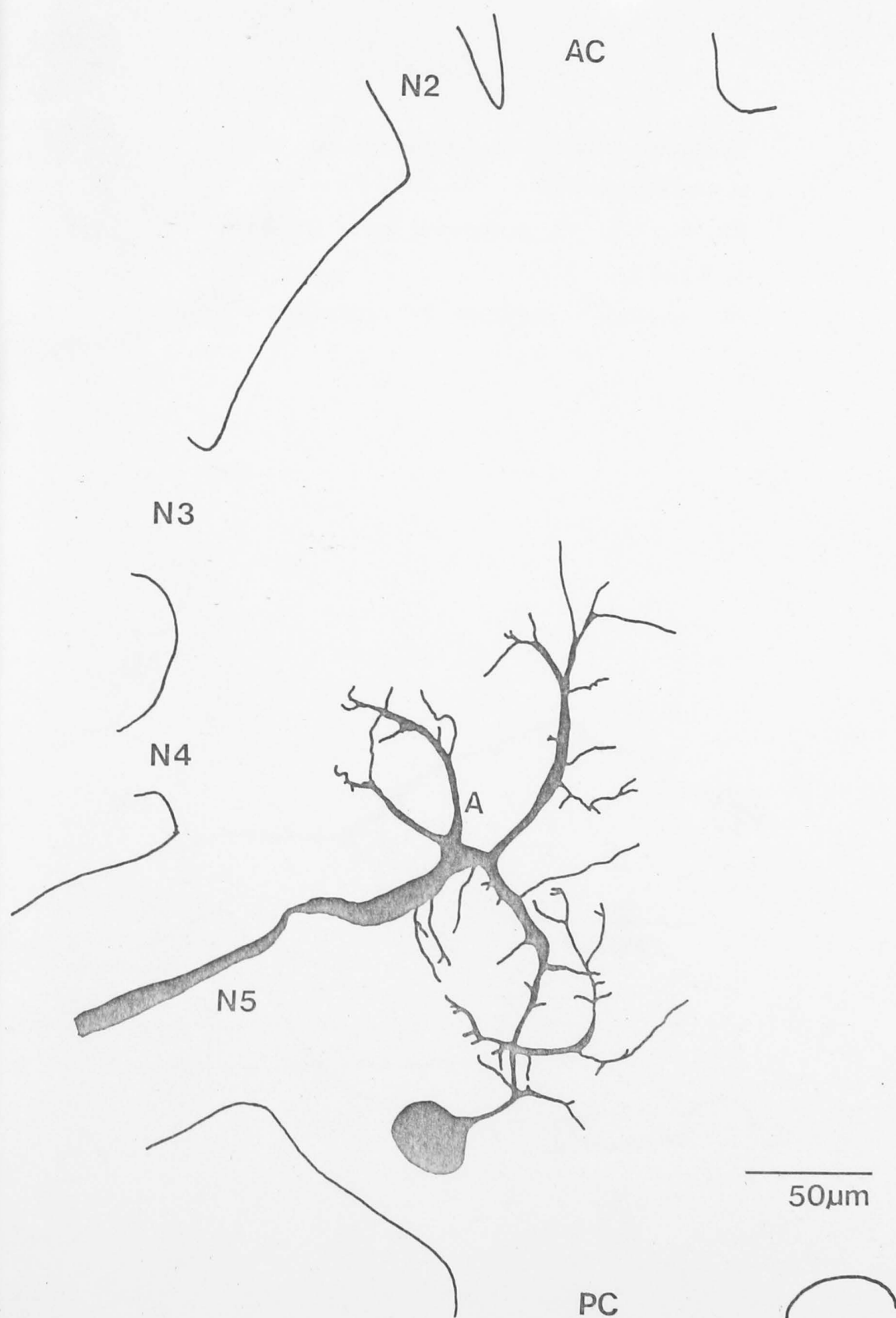


Fig. 13

Dendritic anatomy of motoneuron no. 40 in
control animals.

N2, N3, N4, N5; peripheral nerve trunks 2,
3, 4 and 5.

AC, anterior connective; PC, posterior connective.

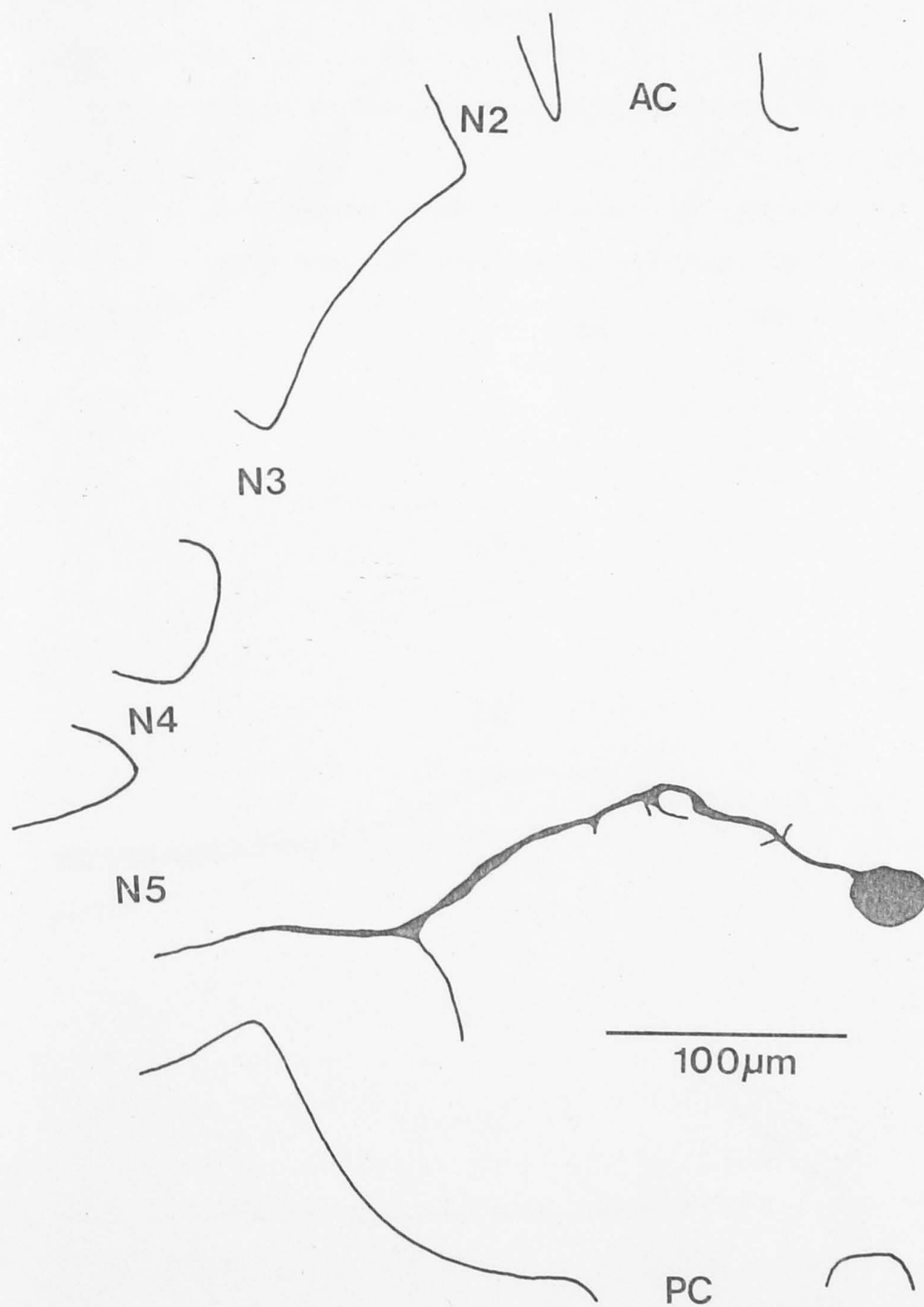
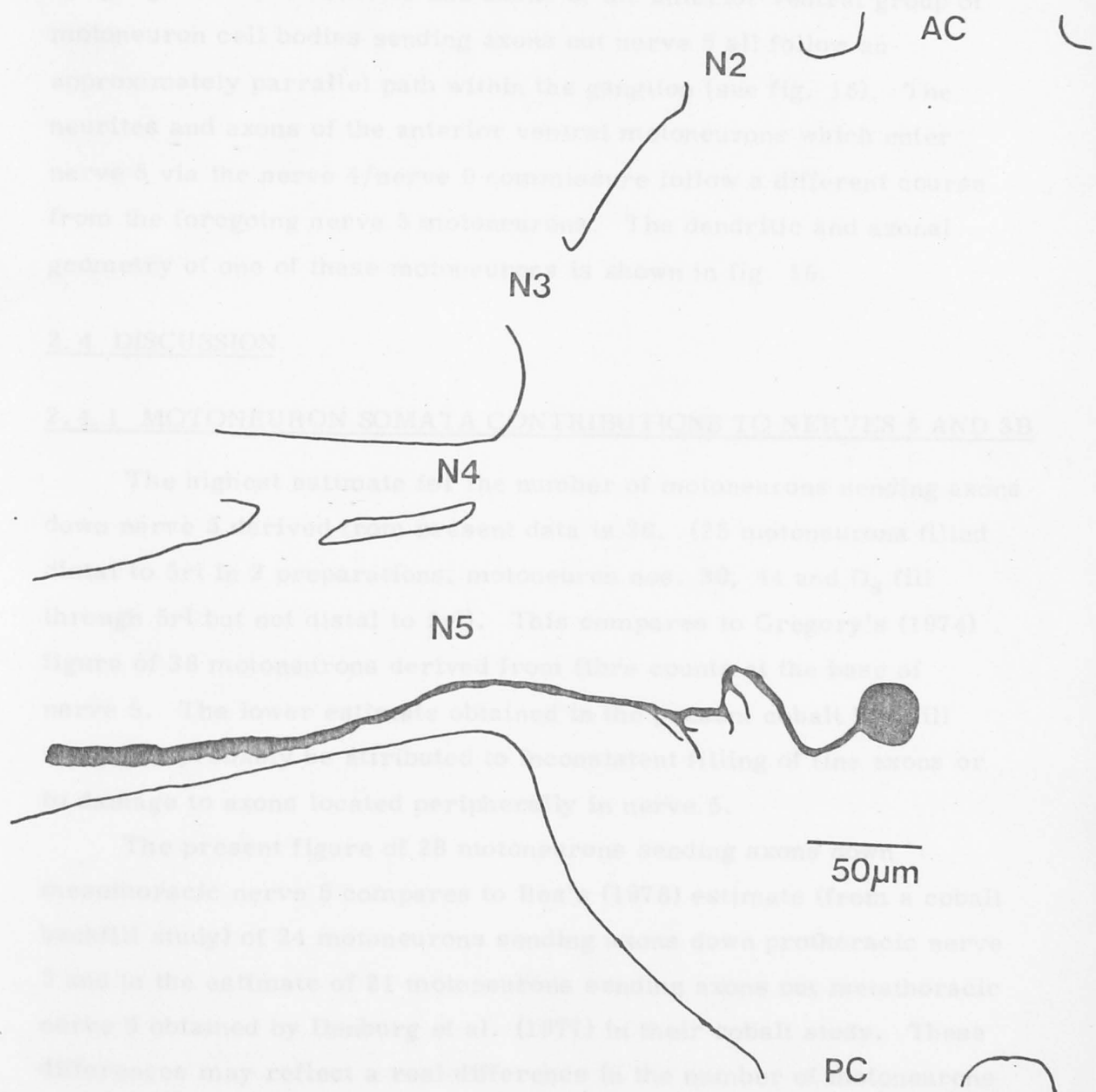


Fig. 14

Dendritic anatomy of motoneuron no. 44 in normal animals.

N2, N3, N4, N5, peripheral nerve trunks 2, 3, 4 and 5; AC, anterior connective; PC, posterior connective.



restricted dendritic branching compared to motoneurons 30 and D_S . The axons of motoneurons 40 and 44 follow a similar course within the ganglion. The neurites and axons of the anterior ventral group of motoneuron cell bodies sending axons out nerve 5 all follow an approximately parallel path within the ganglion (see fig. 15). The neurites and axons of the anterior ventral motoneurons which enter nerve 5 via the nerve 4/nerve 5 commissure follow a different course from the foregoing nerve 5 motoneurons. The dendritic and axonal geometry of one of these motoneurons is shown in fig. 16.

2.4 DISCUSSION

2.4.1 MOTONEURON SOMATA CONTRIBUTIONS TO NERVES 5 AND 3B

The highest estimate for the number of motoneurons sending axons down nerve 5 derived from present data is 28. (25 motoneurons filled distal to 5r1 in 2 preparations; motoneuron nos. 30, 44 and D_S fill through 5r1 but not distal to 5r1). This compares to Gregory's (1974) figure of 36 motoneurons derived from fibre counts at the base of nerve 5. The lower estimate obtained in the present cobalt backfill study can probably be attributed to inconsistent filling of fine axons or to damage to axons located peripherally in nerve 5.

The present figure of 28 motoneurons sending axons down mesothoracic nerve 5 compares to Iles's (1976) estimate (from a cobalt backfill study) of 24 motoneurons sending axons down prothoracic nerve 5 and to the estimate of 21 motoneurons sending axons out metathoracic nerve 5 obtained by Denburg et al. (1977) in their cobalt study. These differences may reflect a real difference in the number of motoneurons sending axons out nerve 5 in the three thoracic ganglia but are more likely to be due to technical differences in filling efficiency. The mesothoracic nerve 5 motoneuron cell bodies identified in this study occupy the same position in the ganglion relative to other cell bodies as do the metathoracic nerve 5 motoneuron cell bodies (using Cohen and Jacklett's (1967) metathoracic cell map).

Fig. 15

Anatomy of neurites and axons of anterior control
nerve 5 motoneuron group.

N2, N3, N4, N5, peripheral nerve trunks 2, 3, 4
and 5; AC, anterior connective; PC, posterior connective.

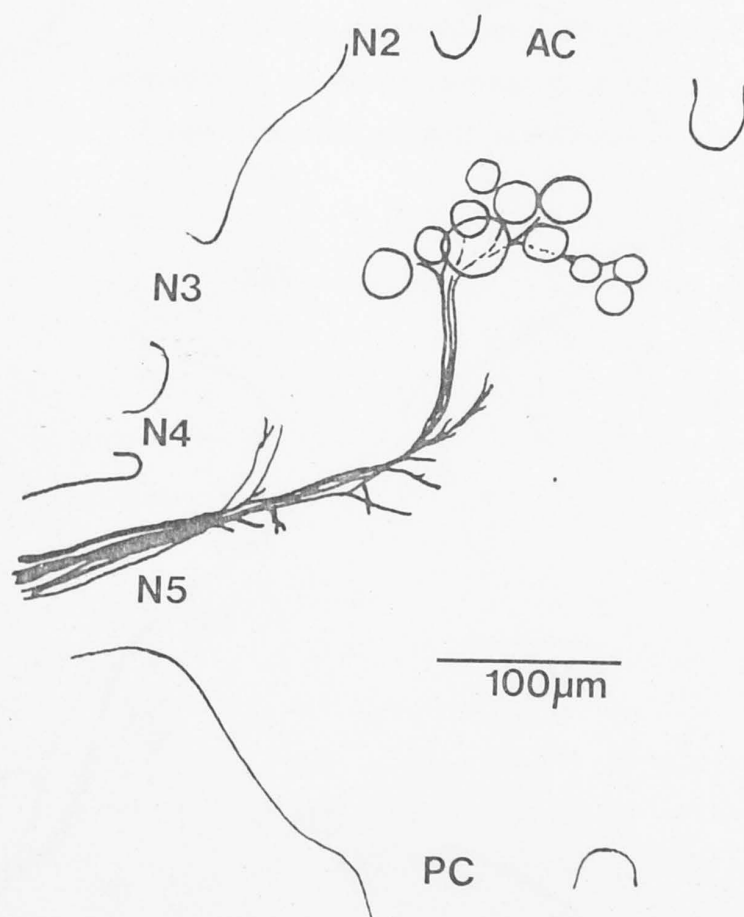


Fig. 16

Dendritic and axonal geometry of a motoneuron
in the anterior ventral nerve 5 motoneuron group
which is located more lateral and anterior to the
main nerve 5 motoneuron group.

N2, 3, 4, 5, nerve trunks 2, 3, 4 and 5;

C, commissure linking nerves 4 and 5.

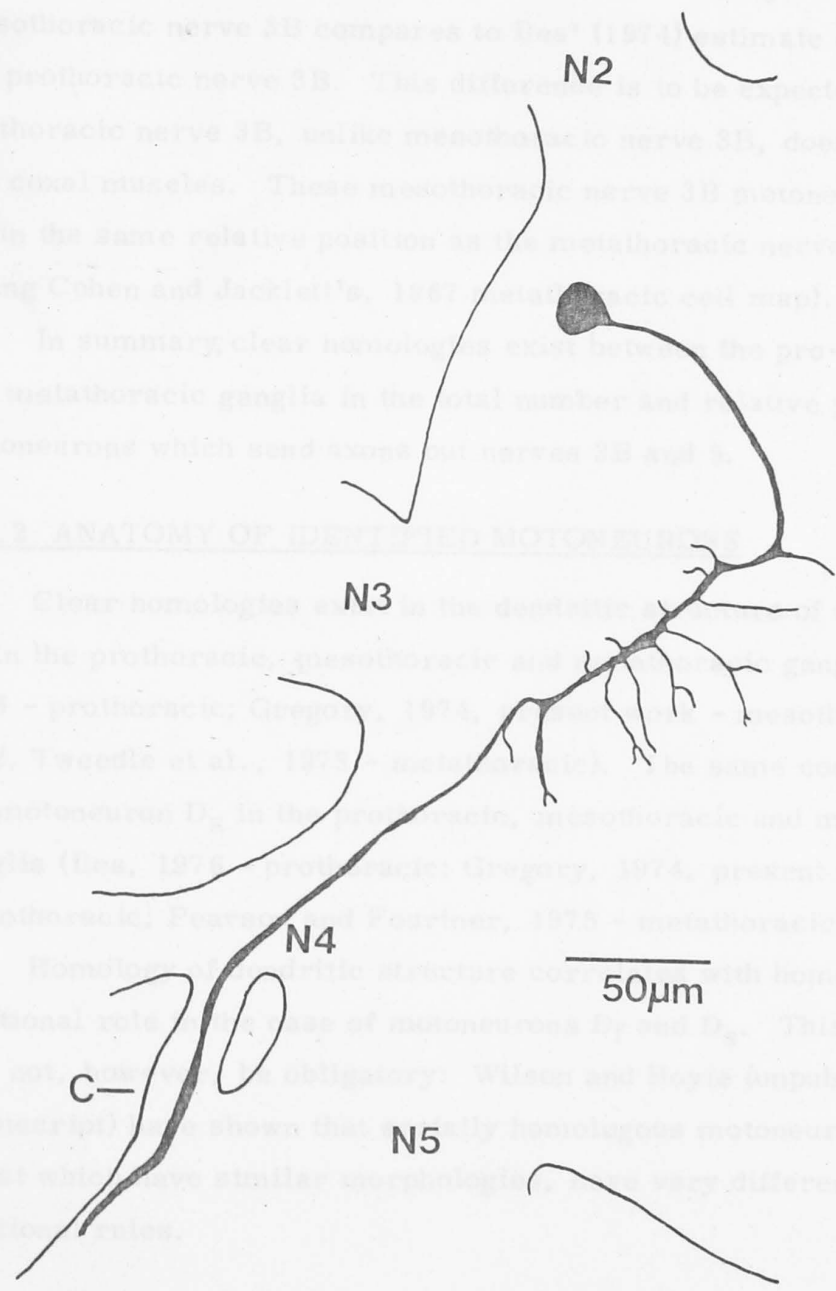
The present estimate of 27 motoneurons innervating axons down mesothoracic nerve 3B compares to Best (1974) estimate of 10 neurons for prothoracic nerve 3B. This difference is to be expected since prothoracic nerve 3B, unlike mesothoracic nerve 3B, does not innervate any axial muscles. These mesothoracic nerve 3B motoneurons contain 11 in the same relative position as mesothoracic nerve 3B somata (using Cohen and Jacklet's, 1977, nomenclature for cell map).

In summary clear homologies exist between the pro-, meso-, and metathoracic ganglia in the total number and relative positions of motoneurons which innervate nerves 3B and 3C.

3.3.3 ANATOMY OF IDENTIFIED MOTONEURONS

Clear homologies exist between the identified motoneurons in D_2 in the prothoracic, mesothoracic and metathoracic ganglia (see, 1974 - prothoracic; Gregory, 1974 - mesothoracic; Best, 1977, Tzeng et al., 1977 - metathoracic). The same correlation holds for motoneuron D_2 in the prothoracic, mesothoracic and metathoracic ganglia (see, 1974 - prothoracic; Gregory, 1974, present work - mesothoracic; Pearson and Pearson, 1973 - metathoracic).

Homologous cells in the structure of the motoneuron in relation to the axonal pattern of motoneuron D_2 . This correlation may not be obligatory: Wilson and Hoyt (unpublished manuscript) have shown that homologous motoneurons in the metathoracic ganglion have similar morphology but very different axonal patterns.



The present estimate of 27 motoneurons sending axons down mesothoracic nerve 3B compares to Iles' (1974) estimate of 10 neurons for prothoracic nerve 3B. This difference is to be expected since prothoracic nerve 3B, unlike mesothoracic nerve 3B, does not innervate any coxal muscles. These mesothoracic nerve 3B motoneuron somata lie in the same relative position as the metathoracic nerve 3B somata (using Cohen and Jacklett's, 1967 metathoracic cell map).

In summary, clear homologies exist between the pro-, meso-, and metathoracic ganglia in the total number and relative positions of motoneurons which send axons out nerves 3B and 5.

2.4.2 ANATOMY OF IDENTIFIED MOTONEURONS

Clear homologies exist in the dendritic structure of motoneuron D_S in the prothoracic, mesothoracic and metathoracic ganglia (Iles, 1976 - prothoracic; Gregory, 1974, present work - mesothoracic; Iles, 1972; Tweedle et al., 1973 - metathoracic). The same conclusion holds for motoneuron D_S in the prothoracic, mesothoracic and metathoracic ganglia (Iles, 1976 - prothoracic; Gregory, 1974, present work - mesothoracic; Pearson and Fournier, 1975 - metathoracic).

Homology of dendritic structure correlates with homology in functional role in the case of motoneurons D_f and D_S . This correlation may not, however, be obligatory: Wilson and Hoyle (unpublished manuscript) have shown that serially homologous motoneurons in the locust which have similar morphologies, have very different functional rules.

INTRODUCTION

The ability of nerves to regenerate after transection is a well-known phenomenon. In the central nervous system, however, the ability to regenerate is limited. The formation of new synapses after nerve injury is a complex process. The formation of new synapses after nerve injury is a complex process. The formation of new synapses after nerve injury is a complex process.

CHAPTER 3

REINNERVATION OF LEGS WITH EXCISED MUSCLES

The purpose of this chapter is to describe the experimental design and results of the study. The study was designed to test the hypothesis that the formation of new synapses after nerve injury is a complex process. The study was designed to test the hypothesis that the formation of new synapses after nerve injury is a complex process.

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The results show that a number of factors can influence the formation of new synapses after nerve injury. The results show that a number of factors can influence the formation of new synapses after nerve injury.

3.1 INTRODUCTION

The identified neurons of invertebrate nervous systems provide us with a powerful tool with which to attack the problem of the genesis of specific neuronal connections. As in the vertebrates, regeneration of neuronal pathways in the adult rather than their formation in the embryo, has received most attention. Two conclusions can be drawn from studies of neural regeneration made in the invertebrates to date:

a) after axotomy, individual motor, sensory and interneurons are capable of re-establishing synaptic connections with their original targets (Pearson and Bradley, 1972; Van Essen and Jansen, 1976; Carbonetto and Muller, 1977).

b) the precision of reinnervation is high: if the findings that regenerating neurons may reinnervate targets which are contralateral (Fourtner et al., 1977; Palka and Edwards, 1974) or segmental (Young, 1972) homologues of their normal targets are excluded, there exists no unequivocal report of innervation of an inappropriate target by a regenerating motoneuron and only one such report for a regenerating sensory neuron (Van Essen and Jansen, 1976).

The possibility exists, however, that misinnervation does occur more widely but that it has been masked by the experimental design of previous studies. The experiments described in this chapter were carried out with a view to "forcing" a motoneuron to innervate an inappropriate muscle. The strategy used was to excise a group of muscles from the leg of an adult cockroach, then to test whether those neurons which normally innervate these excised muscles would innervate nearby denervated muscles. The test was carried out on identified neurons so that regenerated connections could be classified as either correct or incorrect with certainty.

The results show that a number of neurons do make functional connections with incorrect muscles in such operated animals.

3.2 MATERIALS AND METHODS

3.2.1 MUSCLE REMOVAL OPERATION

Adult male cockroaches obtained from the same colony as control animals (see section 2.2.1) were used exclusively in this study. The animal was anaesthetized with CO₂ and restrained, ventral side up, on a wax bottomed operating dish with strips of plasticine. A flap of cuticle was removed from the ventral surface of the coxa of the right mesothoracic leg so as to expose the coxal depressor muscles. Muscles 136, 137, 135d, 135d', 135e and 135e' were excised together with the coxal portions of muscles 135a, b and c and nerve 5 was cut midway down the coxa (fig. 3). It was difficult to avoid removing muscle 138 in the course of this operation.

The basisternum was cut and folded back to expose the mesothoracic ganglion. Nerve 3B was cut approximately 0.5 mm from the ganglion with scissors. In some animals nerves 3A and 3B could not be distinguished: nerve 3 was cut proximal to its bifurcation into 3A and 3B in such cases. The basisternum was then folded back to its original position and the piece of cuticle from the coxa replaced and fixed in position with insect wax.

Operated animals were housed individually in plastic boxes and maintained at 18-26°C with continuous access to water and rat pellets. In some animals nerve 3B was recut three, four or seven weeks later.

The aim of the operation was to favour regrowth of the proximal stump of nerve 5 to the nearby denervated muscle 139c.

3.2.2 ELECTROPHYSIOLOGICAL ASSAY OF NEUROMUSCULAR CONNECTIONS

At varying times after the initial operation the connections of mesothoracic motoneurons to the muscles of the operated leg were assayed electrophysiologically as described in sections 2.2.2 and 2.2.3.

3.3 RESULTS

3.3.1 GENERAL RESULTS OF MUSCLE EXCISION OPERATION: SURVIVAL RATE, SIGNS OF REINNERVATION AND MUSCLE REGENERATION

A survival rate of 35% was obtained for the muscle removal and subsequent nerve recutting operations. However, many of the animals which survived the operation showed degeneration of all muscles in the coxa. Of a total of 143 operated animals it was possible to examine only 32 (22%) electrophysiologically.

In 24 of these 32 animals (75%) muscle 139c showed signs of reinnervation as evidenced by spontaneous or reflex twitching and/or the presence of junction potentials (JP's). Different patterns of electrical activity were recorded from muscle 139c in different individuals during reflex twitching. Invariably, from one to three, small (less than 2 mV) excitatory junction potentials (EJP's) were elicited; often one of these had a tonic pattern of activity. These small EJP's were generally accompanied by one or two larger EJP's (between 5 and 10 mV in size). Fig. 17 shows the amplitude distribution of EJP's in the two animals which showed the most complex patterns of activity. No more than five distinct size classes of EJP's can be distinguished in these animals. More usually no more than three size classes of EJP's could be distinguished. Fig. 18 shows a recording taken from muscle 139c of an experimental animal 138 days after the muscle excision operation in which two small and one large size classes of EJP's can be clearly discerned.

The EJP's recorded in muscle 139c may have resulted from innervation by the correct nerve (3B) and/or by a foreign nerve. Some support for the latter possibility is given by two observations: in six animals nerve sprouts from the proximal stump of nerve 5 were seen heading towards muscle 139c; in two animals EJP's recorded from muscle 139c disappeared when nerve 5 was cut. These findings suggest that muscle 139c may be reinnervated by nerve 5, a foreign nerve, in

Fig. 17

Amplitude distribution of EJP's recorded from muscle 139c in experimental animals C(A) - 45 and C(A) - 18 during reflex twitching (C(A) - 45 examined at 82 days after the operation, C(A) - 18 at 138 days).

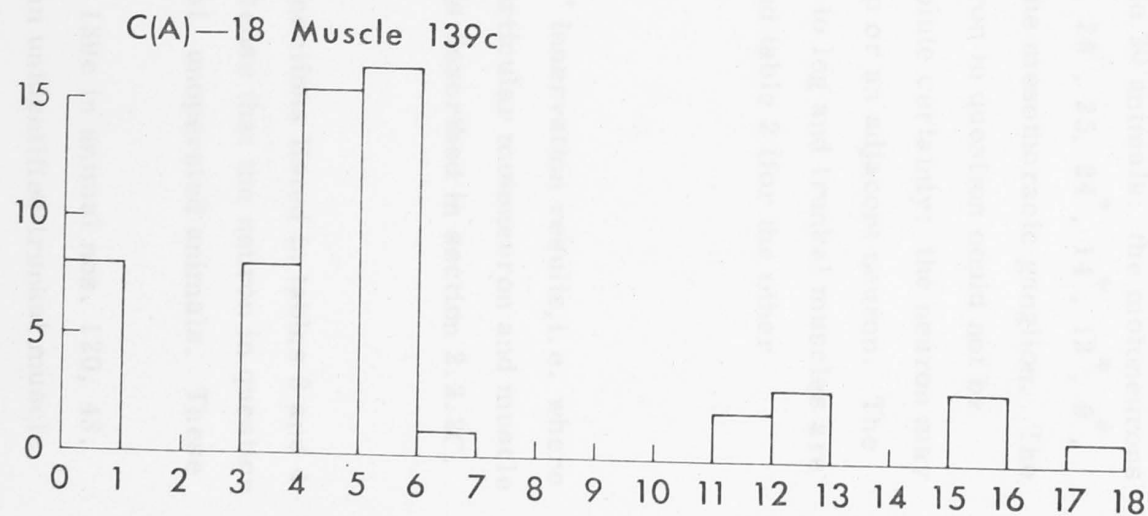
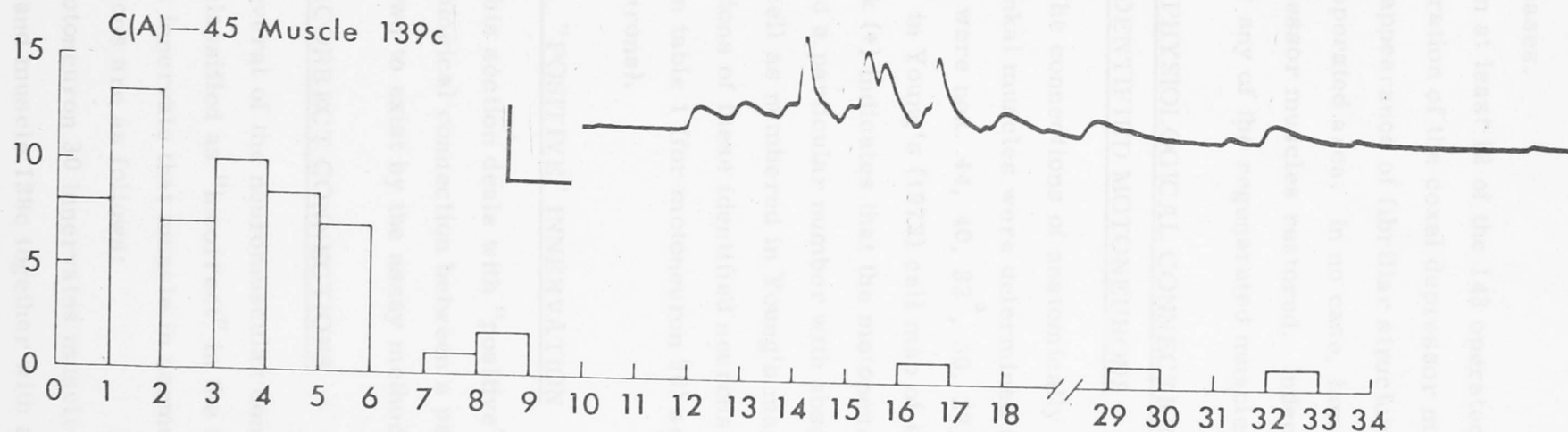
Horizontal axis: size of EJP (Arbitrary units).

Vertical axis: number of EJP's which fall in this size class.

Each histogram is derived from recordings made in a single muscle fibre.

Fig. 18 (Insert)

Intracellular recording from muscle 139c in an animal (C(A) - 18) which had undergone the muscle excision operation 138 days prior to electrophysiological recording, vertical bar 5 mV, horizontal bar 25 msec.



some cases.

In at least 12 of the 143 operated animals, some degree of regeneration of the coxal depressor muscles took place as evidenced by the appearance of fibrillar structures and spontaneous twitching in the operated area. In no case, however, was the normal pattern of depressor muscles restored. Indeed it was not possible to identify any of the regenerated muscles with certainty.

3.3.2 PHYSIOLOGICAL CONNECTIONS OF ANATOMICALLY IDENTIFIED MOTONEURONS

The connections of anatomically identified motoneurons to leg and trunkal muscles were determined in 20 animals: the motoneurons studied were nos. 44, 40, 33^{*}, 30, 27, 26^{*}, 25, 24^{*}, 14^{*}, 12^{*}, 9^{*}, 5^{*}, 18^{*} in Young's (1973) cell map of the mesothoracic ganglion. The asterisk (*) indicates that the motoneuron in question could not be assigned a particular number with absolute certainty: the neuron may be the cell as numbered in Young's map or an adjacent neuron. The connections of these identified neurons to leg and trunkal muscles are shown in table 1 (for motoneuron 30) and table 2 (for the other motoneurons).

3.3.2.1 "POSITIVE" INNERVATION

This section deals with "positive" innervation results, i.e. where a physiological connection between a particular motoneuron and muscle was shown to exist by the assay methods described in section 2.2.2C.

A. INCORRECT CONNECTIONS

Several of the neuromuscular connections listed in tables 3 and 4 can be classified as "incorrect" in the sense that the neuron in question does not innervate that muscle in normal, unoperated animals. These connections are as follows:

- a) Motoneuron 30 innervates muscle 139c in animal nos. 120, 43, 93, 88, and muscle 139c together with an unidentified trunkal muscle in animal no. 141 (table 1).

TABLE 1: Innervation of leg muscles by motoneuron 30 in cockroaches from which coxal depressor muscles had been excised.

Assays: +; twitching of muscle upon firing motoneuron 30 (T) and, in some cases, correlation of JP's recorded from the muscle with motoneuron 30 spikes (C).

-; no visible twitching of this muscle when motoneuron 30 fired (as indicated by twitching of other muscles). (NT).

Abbreviations: N.P.; coxal depressor muscles have not regenerated.

N.P. ?; no obvious regeneration of coxal depressors but could not state definitely because of clotted blood.

Animal No.	Time (days post operation p.o.)	Muscle		
		139c	Regenerated Coxal Depressors	Other
120	43	+ ^T	N. P. ?	- NT
141	47	+ ^{T, C}	N. P.	+ (Trunkal) ^T
43	84	+ ^{T, C}	N. P. ?	- NT
93	116	+ ^T	- NT	- NT
88	253	+ ^T	- NT	- NT
142	51	- NT	N. P. ?	+ (138) ^T
151	148	- NT	N. P.	+ (Trunkal) ^T
98	223	- NT	N. P. ?	+ (Coxal ^T Levator)
97	48	- NT	- NT	+ (Tibial ^T Flexor)
128	36	- NT	+ ^T	- NT
106	39	- NT	+ ^T	- NT
143	60	- NT	+ ^T	- NT
155	65	- NT	+ ^T	- NT
146	67	- NT	+ ^T	- NT

TABLE 2: Innervation of leg muscles by anatomically identified motoneurons in cockroaches from which coxal depressor muscles had been excised.

Assays: +; twitching of muscle when motoneuron fired (T) or correlation of JP's in muscle with motoneuron spikes (C).

-; no visible twitching of this muscle when motoneuron fired (indicated by twitching of other muscles) (NT);

or no correlation between JP's recorded in muscle and motoneuron spikes (N.C.).

*; see text.

Abbreviations: N.P. ?; no obvious regeneration of coxal depressors but could not state this definitely because of clotted blood.

N.R.; recordings were not made from these muscles, therefore it is impossible to say whether they were innervated by inhibitors.

Motoneuron No.	Animal No.	Time (days p.o.)	Muscle		
			139c	Regenerated Coxal Depressors	Other
* 5	143	60	+ T	- NT	- NT
* 9	155	65	+ T	- NT	- NT
* 12	143	60	+ T	- NT	- NT
* 14	155	65	+ C, T	- NT	- NT
* 18	144	156	- NT	+ T	- NT
* 24	148	50	- NT	- NT	+ T (Trunkal)
25	128	36	- NT	+ T	- NT
* 26	93	116	+ T	- NT	- NT
27	128	36	+ T	- NT	- NT
	120	43	+ T	N.P.?	- NT
* 33	106	39	- NT	- NT	+ (Trunkal)
40	43	84	- NC	N.R.	N.R.
	117	48	- NC	N.R.	N.R.
44	43	84	- NC	N.R.	N.R.

b) Motoneuron 30 innervates muscle 138 in animal no. 142 and unidentified trunkal, coxal levator and tibial flexor muscles in animals 151, 98, 97 respectively (table 1).

All of the foregoing connections can be classed as incorrect on the grounds that motoneuron 30 is known to innervate only coxal depressor muscles in normal animals (Pearson and Iles, 1971; Young, 1972).

c) Motoneuron no. 33 innervates an unidentified trunkal muscle in animal no. 106, motoneuron no. 27 innervates muscle 139c in animals 128, 120, motoneuron 26 innervates muscle 139c in animal 93, motoneuron 25 innervates a regenerated coxal depressor muscle in animal no. 128, motoneuron 24 innervates an unidentified trunkal muscle in animal 148, motoneuron 14 innervates muscle 139c in animal 155 and motoneuron 12 innervates muscle 139c in animal 143 (table 2).

All of these connections can be classed as incorrect for the following reasons: motoneurons 33, 27, 26, 25, 24, 14 and 12 do not send axons down nerve 3B in normal animals (see fig. 8) and since nerve 3B alone innervates muscle 139c in normal animals, innervation of muscle 139c by motoneuron nos. 27, 26, 14 and 12 here represents misinnervation; motoneuron no. 33 innervates only the retractor unguis muscle in normal animals (Young, 1973) and so innervation of a trunkal muscle in experimental animal no. 106 represents a mistake; coxal depressor muscles are innervated by only neurons 30, 56, 40, 41 and 44 or nerve 4 neurons in normal animals, so innervation of a regenerated coxal depressor muscle by motoneuron no. 25 in animal no. 128 represents misinnervation; and motoneuron no. 24 sends an axon out nerve 5 (see fig. 7), which innervates only leg muscles in normal animals, so innervation of a trunkal muscle by this neuron in animal no. 148 represents misinnervation. Even though some of the motoneurons in table 2 could not be unidentified unambiguously, the above conclusions are still valid, as, in every case, immediately neighbouring neurons are also "foreign" to the particular muscle innervated.

Several aspects of these results merit special attention: one identified motoneuron, cell 30, innervates different incorrect muscles

including 139c, 138 and unidentified trunkal, coxal levator or tibial flexor muscles, in different animals; conversely, one muscle, 139c, is innervated by different identified motoneurons, including cell nos. 30, 27, 26, 14 and 12, in different animals; motoneurons 33, 27, 26, 25, 24, 14 and 12 innervate incorrect muscles, and not their correct target muscles, even though their correct target muscles (femoral/tibial muscles) are present.

B. INNERVATION OF REGENERATED COXAL DEPRESSOR MUSCLES

The connections of identified motoneurons to regenerated coxal depressor muscles were determined in seven animals: these muscles are innervated by motoneuron no. 30 in animals 155, 106, 128, 143, 146, by motoneuron no. 25 in animal no. 128 and by motoneuron no. 18 in animal no. 144. Innervation by motoneuron no. 25 represents an incorrect connection as discussed in section A above. Motoneuron 30 innervates coxal depressor muscles 136, 137, 135d¹, 135e¹ (Iles, 1972) and motoneuron 18, muscle 135a (Young, 1972) in normal animals. However, innervation of regenerated coxal depressor muscles by these two motoneurons could not be classified as either correct or incorrect as the individual identity of the regenerated muscles could not be established.

C. TIME COURSE OF INNERVATION

There are no obvious trends in the pattern of neuromuscular connections with time. For example, misinnervation of muscle 139c occurs as early as 43 days post operation (p.o.) (by cell 30 in animal no. 120) and as late as 253 days p.o. (by cell 30 in animal no. 88).

3.3.2.2 "NEGATIVE" INNERVATION

While it is clear that anatomically identified motoneurons do make physiological connections with muscles in the operated leg, it is equally apparent that a given motoneuron does not innervate all

muscles in the leg nor is a given muscle innervated by all motoneurons. This section deals with cases where a motoneuron was found not to innervate a particular muscle or muscles. These "negative" innervation results must be interpreted with caution. As discussed in section 2.2.2C the methods used in this study to assay absence of innervation may be unreliable under some circumstances.

a) With one exception (motoneuron 30 innervating muscle 139c and an unidentified trunkal muscle simultaneously in animal no. 141), no convincing evidence was obtained to suggest that one motoneuron may innervate more than one muscle in any individual animal, (tables 1 and 2). This result may be due to the nature of the assay used; absence of an observable twitch in a muscle was scored as negative innervation of that muscle by the neuron in question. Functional synaptic connections on small numbers of muscle fibres or on "slow" muscle fibres may have gone undetected by this assay.

b) No convincing evidence was obtained to suggest that a regenerating motoneuron may innervate a muscle which has not been previously denervated. Indeed in 21 out of 27 cases (78%) where positive innervation was found, the motoneuron in question clearly innervates a previously denervated muscle. (Motoneurons 30, 27, 26, 14, 12, 9 and 5 innervate muscle 139c ($n = 12$), motoneurons 30, 25 and 18 innervate regenerated coxal depressor muscles ($n = 7$), motoneuron 30 innervates muscle 138 ($n = 1$) and an unidentified tibial flexor muscle ($n = 1$). In the remaining six cases (motoneurons 33, 30, 24 and 12 innervate unidentified trunkal muscles ($n = 5$) and motoneuron 30 innervates an unidentified coxal levator muscle ($n = 1$)), the possibility exists that the nerves originally supplying the muscles innervated by the regenerating motoneurons may have been cut at the time of the muscle excision operation.

c) No more than five distinct sizes of junction potentials were recorded from muscle 139c in individual operated animals during spontaneous or reflex muscle activity; generally no more than three distinct JP sizes were recorded, (see section 3.3.1).

Assuming that different motoneurons elicit JP's of different amplitude in an individual muscle fibre, and that most or all neurons innervating a muscle are active during reflex activity, the previous result suggests that only a small fraction of the total population of ca. 28 axotomized nerve 5 motoneurons (see page 27) innervates muscle 139c at any one time. Neither of the assumptions made here was tested in the present study and thus the limit of 5 motoneurons may represent an underestimate of the number of neurons actually innervating 139c. Nevertheless, it seems likely that more classes of JP's would have been detected if all 28 neurons had innervated 139c.

d) Using the criterion of absence of correlation between JP's recorded in the muscle with intracellularly recorded soma spikes, the inhibitory motoneurons 44 and 40 have been found not to innervate muscle 139c in one animal (no. 43) and two animals (nos. 43, 117) respectively. Furthermore inhibitory junction potentials (IJP's) were recorded from muscle 139c during spontaneous or reflex activity in only one of 32 animals examined.

3.3.2.3 FREQUENCY OF OCCURRENCE OF PARTICULAR NEUROMUSCULAR CONNECTIONS

Some information was obtained in this study regarding the frequencies with which particular motoneurons innervate different muscles in separate animals:

a) Of eight animals in which the coxal depressor muscles show signs of regeneration, cell 30 innervates muscle 139c in two cases (animal nos. 116, 253), an unidentified tibial flexor muscle in one case (animal no. 97) and regenerated coxal depressors in five cases (animal nos. 155, 106, 128, 143, 146).

b) Of five animals showing no signs of regeneration of the coxal depressor muscles, cell 30 innervates muscle 139c in two cases (animal nos. 93, 88) and muscle 138, unidentified trunkal muscles and coxal levator muscles in animals 142, 151 and 98 respectively.

c) Motoneuron 27 innervates only muscle 139c in the two animals (nos. 128, 120) in which the connections of this neuron were determined. Motoneurons 26, 14 and 12 innervate only muscle 139c in single cases in which connections of these neurons were assayed (animals 93, 155 and 143 respectively).

3.4 DISCUSSION

3.4.1 CAN INVERTEBRATE MOTONEURONS INNERVATE FOREIGN MUSCLES?

Previous studies in the invertebrates have indicated that regenerating motoneurons reform connections with their original muscles (leech - Van Essen and Jansen, 1976; cockroach - Pearson and Bradley, 1972; Westin and Camhi, 1975; crayfish - Bittner, 1973) or with contralateral (Fourtner et al., 1977) or segmental (Young, 1972) homologues. The specificity of reinnervation appears to be high: individual motoneurons have not been previously reported to make incorrect functional connections with muscles.

This result suggests the following rule which, by itself, could lead to specific reinnervation: "invertebrate motoneurons may only innervate appropriate target muscles". The present finding that regenerating cockroach motoneurons are capable of innervating foreign muscles suggests that this rule is not valid for at least some cockroach motoneurons and therefore cannot, by itself, account for specific reinnervation in the cockroach.

The foregoing conclusion is qualified because of the possibility that removal of a group of muscles from the leg induces "respecification" of the remaining muscles, such that the coxal levator muscle 139c, for example, acquires the identity of a coxal depressor muscle. Motoneuron 30 may innervate muscle 139c in an operated animal while such a connection may be "disallowed" in an unoperated animal. The present study may therefore say little about rules for connectivity in a normal animal. (Respecification of target cell

identity has been advanced as a possible explanation of the finding in Xenopus that retinal fibres from the nasal half of an eye can project in topographic order to a complete optic tectum (Meyer and Sperry, 1973; Yoon, 1975)).

3.4.2 COMPARISON WITH OTHER INVERTEBRATE STUDIES

The present results represent the first unequivocal report of misinnervation by regenerating invertebrate motoneurons. Several possible explanations can be advanced as to why such misinnervation has not been reported earlier.

Firstly, the methods used to assay neuromuscular connections in many previous studies do not allow one to be confident that functional misinnervation has occurred. For example Bodenstein (1955, 1957) reported that gross nerve connections are seen, in the cockroach, between the anterior and posterior connectives and metathoracic legs in animals from which the metathoracic ganglion has been excised. Guthrie and Banks (1968) found that ganglia transplanted into the coxae of adult cockroaches make connections with leg muscles through their connectives. In neither of these cases, however, was it shown that the abnormal nerve connections were functional. Furthermore the muscles in question may have been innervated by correct motoneurons which had regrown to the periphery via incorrect nerve roots. Denburg et al. (1977) showed, in a cobalt backfill study of the coxal depressors of the cockroach, that following a nerve crush, some motoneurons do send axons down a nerve branch to inappropriate muscles. However, the question of whether these neurons made functional connections on those foreign muscles was not addressed. Kennedy and Bittner (1974) reported that abnormal reflex behaviour developed after excising a length of motor nerve in the crayfish while Sahota and Edwards (1969) found that supernumerary legs transplanted to the mesothorax of the cricket develop uncoordinated movements. In neither case, however, was it confirmed that the abnormal behaviour resulted from incorrect innervation.

Secondly, the experimental design used in previous studies may have been biased towards achieving specific reinnervation. Pearson and Bradley (1972) and Van Essen and Jansen (1976) investigated the specificity of reinnervation after cutting and crushing motor nerves. In both cases the regenerating motoneurons may have been guided back to their former target muscles by the distal nerve sheaths (but see chapter 5). It may even be necessary to deprive neurons of the opportunity of innervating their correct targets in order to cause misinnervation.

Thirdly, the emphasis in most regeneration studies has been on demonstrating that motoneurons can establish connections with their original targets: the possibility that such neurons may have also made connections with incorrect muscles has not been rigorously tested.

Finally, most regeneration studies to date have only examined late stages of the regeneration process in detail: e.g. Pearson and Bradley (1973) and Young (1972) examined their animals 5 months post operatively. Early, transient functional misinnervation may have been overlooked.

3.4.3 RESTRICTIONS ON FUNCTIONAL SYNAPSE FORMATION

This study demonstrates that regenerating cockroach motoneurons are capable of forming functional synaptic connections with muscles other than their appropriate ones. This result raises the question of whether the outgrowing axon tip of an individual motoneuron may innervate all muscles it encounters or whether there are restrictions on which muscles may be innervated. Several findings in this study suggest that a motoneuron does not innervate targets at random and provide evidence for two types of restrictions on synapse formation: general and specific.

3.4.3.1 GENERAL RESTRICTIONS

General restrictions are defined as those restrictions on the formation of a functional connection between a muscle and motoneuron which apply irrespective of the identities of the muscle and motoneuron

involved. Two such restrictions are suggested by the present study:

- (1) A regenerating motoneuron may innervate only one muscle at a time.
- (2) There are limits to the number of neurons which may innervate a muscle at one time.

Restriction (1) is suggested by the finding that regenerating motoneurons innervate more than one muscle in only 4% of animals examined. The possible flaws in this interpretation of the physiological data have been discussed on page 39. Furthermore, it would need to be verified that an individual regenerating motoneuron did have physical access to more than one muscle before this possible restriction acquired more validity. This restriction cannot be absolute for regenerating cockroach motoneurons since Pearson and Bradley (1972) have reported that motoneuron 30 reinnervates all four of its normal target muscles during regeneration to an intact leg. Further, this restriction cannot apply to innervation during embryogenesis as many cockroach motoneurons innervate more than one muscle in normal adults (chapter 2).

Nonetheless, present results, e.g. that motoneuron no. 30 does not innervate more than one of muscles 139c, 138, regenerated coxal depressors, trunkal or coxal levator muscles in any one individual, whereas it innervates each of these muscles in separate individuals, argue for some type of restriction on the number of muscles that a regenerating cockroach motoneuron is capable of innervating at one time.

Restriction (2) is inferred from the finding that only a small fraction of the total population of ca. 28 axotomized nerve 5 neurons innervate muscle 139c at any one time. This conclusion, as pointed out on page 40, rests upon assumptions which remained untested.

A further restriction, which has been investigated in two previous regeneration studies in the cockroach, is that regenerating motoneurons only innervate muscles which have been previously denervated. Jacklett and Cohen (1967) reported that ganglia implanted into the legs

of cockroaches only develop connections with denervated host muscle. In contrast Guthrie and Banks (1968) claim that such ganglia innervate any muscle fibre in their vicinity. No evidence was obtained in the present study to suggest that a motoneuron may innervate a muscle which has not been previously denervated. However, neither can this possibility be excluded from the present results. Thus the question of whether regenerating cockroach motoneurons are capable of innervating muscles with an intact nerve supply remains unanswered.

3.4.3.2 SPECIFIC RESTRICTIONS

Specific restrictions are defined as those restrictions on the formation of a functional connection which apply to specific motoneuron-muscle combinations. Such specific restrictions may reveal the rules for the formation of functional connections (see section 1.5).

Two candidate "rules" can be eliminated by findings of misinnervation of muscles by identified motoneurons in the present study:

- (1) A motoneuron cannot innervate muscles antagonistic to its correct targets. This rule is eliminated since cell 30, a coxal depressor motoneuron, innervates coxal levator muscles.
- (2) A motoneuron cannot innervate muscles in a foreign segment of the leg. This rule is negated by the findings that motoneuron no. 30 innervates femoral and trunkal muscles and that several femoral/tibial motoneurons innervate 139c, a coxal muscle.

On the contrary, other candidate rules are supported by the failure to find connections between certain identified motoneurons and muscles (section 3.3.2.2) and by data on the frequency of occurrence of particular motoneuron-muscle connections (section 3.3.2.3):

- (1) The rule, inhibitory motoneurons may not innervate muscle 139c, is suggested by the finding that IJP's were rarely recorded from muscle 139c, and that inhibitory motoneurons 40 and 44 did not innervate muscle 139c in any of the three cases in which these connections were assayed as determined by lack of correspondence of motoneuron

soma spikes with muscle JP's.

(2) Motoneuron 30 innervates coxal depressor muscles in 5 out of 8 animals in which the depressors have regenerated. This result may either suggest the rule "motoneuron 30 tends to innervate its correct muscles", or the rule "motoneuron 30 tends to innervate coxal depressor muscles rather than, say, coxal levator muscles", depending on the identity of the depressor muscles innervated. There is no clear indication that cell 30 innervates one muscle in preference to another in animals in which the coxal depressors have not regenerated. However, only a small number of animals (5) were studied; preferences might become apparent with a larger sample of animals.

(3) Several other identified motoneurons are found not to innervate particular muscles, e.g. motoneurons 27, 26, 14 and 12 innervate muscle 139c and not regenerated coxal depressors which are also present. However, because there are only one or two observations in each case one cannot draw generalisations such as "motoneurons 27 and 14 may not innervate depressor muscles".

It only becomes valid to infer rules for connectivity from "negative" or "preferred" innervation data if it can be shown that the "forbidden" or "less-favoured" muscles are accessible to the neuron in question, i.e. that the motoneuron is not prevented from innervating these muscles for reasons such as: a) the motoneuron does not grow towards the muscle because of physical barriers or because it cannot follow the path to the muscle (see section 5.3.1), b) the "forbidden" muscles are not denervated or are fully occupied (see section 5.3.2.1). Furthermore, the conclusion that a motoneuron is not able to innervate a particular muscle must be drawn from many trials in which that motoneuron is challenged to innervate that muscle.

In general, all of those conditions have not been met in this study. Nevertheless, this approach is a potentially valuable one; by noting the response of a motoneuron when challenged to innervate specific muscles in repeated trials we may hope to obtain a catalogue of "acceptable" or "preferred" muscles for that neuron. Such

information may shed light on the rules according to which specific neuromuscular connections are made.

3.4.4 MISINNERVATION IN THE PRESENCE OF CORRECT TARGETS

A surprising result is that a number of femoral/tibial neurons innervate (foreign) coxal and trunkal muscles to the exclusion of their normal target muscles which are present.

Two possible explanations are: a) the axons of these motoneurons (contained in nerve 5) were still in the process of regrowing to the femur and tibia at the time their connections were assayed. However, the fact that these mistakes are found as late as 116 days after the muscle excision operation makes this seem unlikely; b) these neurons are unable to grow beyond the coxa because of some physical barrier to the growth of nerve 5. In at least one animal, however, motoneuron 30, a nerve 5 neuron, made connections with femoral muscles indicating that there is no physical block to nerve 5 motoneurons per se.

Clearly this phenomenon awaits a satisfactory explanation.

4.1 INTRODUCTION

The work described in chapter 3 enables us to draw several conclusions about the rules for connectivity in a leg from which a group of muscles has been excised. However, because of the possibility of re-specification (see page 41), these rules may not apply to motoneurons regenerating to an intact, unoperated leg. Furthermore, the muscle efference study sheds little light on the question of when crucial decisions are made during neuronal development (see page 107). A more complete study, following regeneration from its earliest stages to the completion, is required if we hope to answer this question.

CHAPTER 4

With the above considerations in mind I have undertaken a study of the course of REINNERVATION OF MUSCLES IN AN INTACT LEG FOLLOWING NERVE CRUSH

4.2 MATERIALS AND METHODS

4.2.1 NERVE CRUSH OPERATION

Adult male cockroaches, obtained from the same colony as control animals, were used in these experiments.

Animals were anaesthetized with CO₂ and restrained ventral side up on a wax-bottomed operating dish. Nerve 2 is visible beneath the transparent arthrodial membrane at the base of the prothoracic leg. A small hole was made in this membrane, a pair of fine forceps inserted and nerve 2 crushed approximately 1 mm from the ganglion. All operations were done on the right nerve. Operated animals were housed individually and maintained at 18-25°C. Nerve 2 was crushed rather than cut to increase the percentage of successful regenerates and to reduce variability in the pattern of regeneration. Dooling et al. (1977) have confirmed that this crushing procedure physically disrupts axons in the crushed region.

4.1 INTRODUCTION

The work described in chapter 3 enables us to draw several conclusions about the rules for connectivity in a leg from which a group of muscles has been excised. However, because of the possibility of respecification (see page 41), these rules may not apply to motoneurons regenerating to an intact, unoperated leg. Furthermore, the muscle excision study sheds little light on the question of when crucial decisions are made during neuronal development (see page 19). A time course study, following regeneration from its earliest stages to its completion, is required if we hope to answer this question.

With the above considerations in mind I have undertaken a time course study of regeneration of motoneurons to a cockroach leg possessing its full complement of muscles.

4.2 MATERIALS AND METHODS

4.2.1 NERVE CRUSH OPERATION

Adult male cockroaches, obtained from the same colony as control animals, were used in these experiments.

Animals were anaesthetized with CO₂ and restrained ventral side up on a wax-bottomed operating dish. Nerve 5 is visible beneath the transparent arthrodial membrane at the base of the mesothoracic leg. A small hole was made in this membrane, a pair of fine forceps inserted and nerve 5 crushed approximately 1 mm from the ganglion. All operations were done on the right nerve. Operated animals were housed individually and maintained at 18-26°C. Nerve 5 was crushed rather than cut to increase the percentage of successful regenerates and to reduce variability in the pattern of regeneration. Denburg et al. (1977) have confirmed that this crushing operation physically interrupts axons in the crushed region.

4.2.2 ELECTROPHYSIOLOGICAL ASSAY

The connections of individual motoneurons to leg muscles were assayed at various times after nerve 5 crush using the electrophysiological techniques described in chapter 2. In addition, in this series of experiments, extracellular recordings of spike activity were made from nerve 5 with a pair of silver hooks (see fig. 5). In cases when spikes recorded intracellularly from a particular motoneuron could be clearly correlated with spikes recorded extracellularly from nerve 5, spike activity in this motoneuron could, from then on, be monitored by nerve 5 recordings alone.

4.2.3 CELL MARKING

In general, motoneuron cell bodies were filled with Procion Brown as described in section 2.2.3. However, horseradish peroxidase (HRP) was used in some preparations in an attempt to fill the axon of the injected cell beyond the site of crushing. Electrodes were filled with a 4% solution of HRP in 0.2 M KCl and driven into the motoneuron cell body (see section 2.2.2). The dye was iontophoretically ejected from the microelectrode by passing one 100 msec., 5 to 100 nA pulse every 300 msec., for a total duration of 10 to 20 minutes. The ganglion was excised 2 to 7 hours after injection and washed in cockroach saline. The presence of HRP was demonstrated by placing the ganglion in a 10% solution of Diamino-benzidine (D.A.B.) in 0.2 M KCl for 30 minutes, washed in saline (La Vail and La Vail, 1972), fixed in Bodian's No. 2 fixative, dehydrated in an alcohol series and cleared in xylene. The injected axon was viewed and drawn as a whole mount.

4.2.4 COBALT BACKFILLS

The cell bodies of motoneurons sending axons down nerve 5r1 were marked by the cobalt backfilling technique described in section 2.2.4. After fixation, dehydration and clearing the ganglion was mounted in Permount and a map drawn of filled and unfilled cell bodies.

4.3 RESULTS

4.3.1 GROSS ASPECTS OF MORPHOLOGICAL AND FUNCTIONAL REGENERATION OF NERVE 5

The nerve 5 crush operation was performed on a total of 288 animals of which 80-90% survived for at least two weeks following the operation. Immediately after crushing, the nerve appeared flattened at the crush site. Up to two post-operative weeks later nerve 5 appeared thinner at the crush site than on either side of the crush while from three weeks post crush the crushed region was thicker than the surrounding nerve. Fine nerve branches were occasionally seen emanating from the crushed site but these could not be traced to their final destinations. Nerve 5, in the region from the crush site to the ganglion, often appeared thinner in operated animals than in controls.

Nerve 5 showed no morphological signs of regeneration in three animals (at 27, 33 and 214 days post crush) out of 61 animals (5%) studied: the proximal end of nerve 5 lay free in the haemocoel or abutted onto the arthrodial membrane. One or both of the following two assays was used to test for functional reinnervation of leg muscles by nerve 5 in regenerates: 1) twitching of leg muscles on stimulating nerve 5 with hook electrodes; or 2) correlation between junction potentials in leg muscles with spikes recorded extracellularly from nerve 5 or intracellularly from known nerve 5 motoneurons. Using these assays regeneration was scored as successful in 10 out of 16 animals (63%) examined at 17-28 days post crush, in 15 out of 18 animals (83%) at 29-43 days post crush, and in 24 out of 27 (89%) at 44-228 days post crush (table 3). These percentages may represent underestimates of the degree of successful regeneration of nerve 5: it is possible that nerve 5 had formed functional connections with muscle fibres which were not recorded from, in some of the 11 animals in the "not scored" column of table 3. Assuming that regeneration of nerve 5 was unsuccessful in these 11 animals, however, the present figures

show that: nerve 5 regenerates in a high percentage of animals (at least 49/61 - 81%); that the percentage of successful regenerates increases with time; and that unsuccessful regenerates are still found as late as 214 days post crush.

In 8 out of 60 operated animals (13%) examined, the coxal depressor muscles 136, 137 showed clear signs of atrophy; fat deposits were often seen surrounding such muscles. Four of these animals were examined prior to 36 days post crush (at 22, 23, 26 and 35 days); in two of these the atrophied muscle 137 was functionally innervated. Electrical activity was not, however, found in atrophied muscles in any of the four animals examined later than 36 days post crush (at 60, 64, 214 and 228 days). Clear signs of atrophy were not observed in the other coxal depressor muscles 135d, d', e, e' in any animals.

4.3.2 WALKING BEHAVIOUR IN OPERATED ANIMALS

Operated cockroaches showed signs of limping with the operated leg in 9 out of 43 cases (animals examined at 12, 17, 26, 27, 28, 30, 32, 34 and 49 days post crush). No obvious limping was apparent in the remaining 34 animals examined from 18 to 222 days post crush. At least 14 of these animals were, however, unable to grasp a wire mesh with the tarsus of the operated leg. In two of these 14 animals, nerve 5 had clearly not regenerated, while in the remaining 12, one or more motoneurons had failed to innervate normal target muscles or had innervated inappropriate muscles (see section 4.3.3).

4.3.3 INNERVATION OF COXAL DEPRESSOR MUSCLES

The connections of motoneurons to the denervated coxal depressor muscles 136, 137, 135d, 135d', 135e, 135e' were examined physiologically in 61 operated animals from 12 to 228 days post crush. Using the criteria of the presence of junction potentials or twitching in response to stimulation of nerve 5 in at least one of these muscles as an assay for functional innervation, this group of muscles was found to be innervated in 47 out of these 61 animals (77%) (table 4).

TABLE 3: Regeneration of nerve 5 to leg muscles at various times after crushing nerve 5.

Assays: +; either twitching of leg muscles on stimulating nerve 5 or junction potentials recorded from leg muscles correlated with spikes in nerve 5 or nerve 5 motoneurons.

-; no morphological signs of regeneration.

Not scored; no activity recorded from muscles examined and/or no twitching when nerve 5 stimulated.

Time (days after crushing N5)	No. of animals		
	+	-	Not Scored
12-28	10	1 (27 days)	5
29-43	15	1 (33 days)	2
44-228	24	1 (214 days)	2
Total	49	3	9

TABLE 4: Innervation of coxal depressor muscles at various times after crushing nerve 5.

Assay: +; either presence of junction potentials in at least one muscle or twitching of at least one muscle on stimulation of nerve 5.

No activity; no junction potentials recorded from muscles examined (not necessarily all coxal depressor muscles).

Time
(days after
crushing nerve 5)

No. of animals

	+	No activity
12-28	9	7
29-43	15	3
44-228	23	4
Total	47	14

4.3.3.1. INNERVATION OF COXAL DEPRESSORS BY MOTONEURON 30

A. INNERVATION OF MUSCLES AS A GROUP

Motoneuron 30 forms functional connections with at least one coxal depressor muscle in 37 out of 43 animals (86%) examined (table 6). Functional connections between motoneuron 30 and coxal depressor muscles were first detected at 30 days post crush and the percentage of animals in which such connections were found increased from 5/13 (38%) at 30-33 days post crush to 9/14 (64%) at 39-43 days post crush. From 44-227 days motoneuron 30 had innervated at least one coxal depressor muscle in 25% of animals assayed.

In two animals at 33-35 days, 9 animals at 39-43 days and 11 animals at 44-228 days it could not be positively determined whether or not motoneuron 30 had innervated a coxal depressor muscle. There is, however, no evidence to suggest that regeneration may have been more successful in these untested animals than in the corresponding scored groups. Therefore it seems reasonable to suggest that from the present

The same assay was applied to each individual coxal depressor muscle to test whether each of these muscles is reinnervated with equal probability (table 5). Functional reinnervation had definitely taken place in muscle 137 in 36 out of 48 animals examined (75%), in muscle 136 in 7 out of 8 animals (87%), muscle 135d in 19 out of 24 cases (80%), in muscle 135d' in 19 out of 20 cases (95%), in muscle 135e in 20 out of 33 cases (61%), and muscle 135e' in 21 out of 27 cases (80%).

The identity of the motoneurons eliciting junction potentials in the coxal depressors was determined by intracellular recording from motoneuron somata and/or extracellular recording from nerve 5. The coxal depressor muscles 137, 136, 135d, 135d', 135e, 135e' in regenerates were found to be innervated by the normal nerve 5 coxal depressor motoneurons 30, D_S, 40, 41, 44 (see chapter 2) and also by unidentified foreign neurons.

4.3.3.1 INNERVATION OF COXAL DEPRESSORS BY MOTONEURON 30

A. INNERVATION OF MUSCLES AS A GROUP

Motoneuron 30 forms functional connections with at least one coxal depressor muscle in 27 out of 43 animals (63%) examined (table 6). Functional connections between motoneuron 30 and coxal depressor muscles were first detected at 20 days post crush and the percentage of animals in which such connections were found increased from 5/13 (38%) at 20-28 days post crush to 9/14 (64%) at 29-43 days post crush. From 44-227 days motoneuron 30 had innervated at least one coxal depressor muscle in 93% of animals assayed.

In two animals at 12-28 days, 6 animals at 29-43 days and 11 animals at 44-228 days it could not be positively determined whether or not motoneuron 30 had innervated a coxal depressor muscle. There is, however, no evidence to suggest that regeneration may have been more successful in these unscored animals than in the corresponding scored groups. Therefore it seems reasonable to suggest from the present

TABLE 5: Innervation of individual coxal depressor muscles
after crushing nerve 5 (all post operative stages
combined).

Assay: +; either presence of junction potentials in at
least one muscle or twitching of at least one
muscle on stimulation of nerve 5.

No activity; no junction potentials recorded
from this muscle.

Muscle	No. of animals	
	+	No activity
137	36	12
136	7	1
135d [/]	19	1
135e [/]	21	6
135d	19	5
135e	20	13

TABLE 6: Innervation of at least one coxal depressor muscle by motoneuron 30 at various times after crushing nerve 5.

Assays: +; correlation of motoneuron 30 spikes with junction potentials from at least one coxal depressor muscle or twitching of at least one coxal depressor muscle upon firing motoneuron 30.

-; no twitching of coxal depressor muscles when motoneuron 30 fired or, no morphological regeneration of N5. (Note that the former assay only indicates failure of motoneuron 30 to innervate fast muscles; innervation of slow muscles may not be detected by this assay).

Time
(days after
crushing nerve 5)

No. of animals

REINNERVATION OF INDIVIDUAL COXAL DEPRESSOR

	+	-
17-19	0	2
20-28	5*	8
29-43	9	5
44-227	13	1
Total	27	16

*: first positive result at 20 days post crush.

These figures suggest that motoneuron 30 innervates the appropriate connections as frequently as correct connections alone correct muscles 137, 136, 135d and 135e are innervated in 38 out of 51 cases (71%) while foreign muscles 135d, 135e are innervated in 4 out of 6 cases (67%). This conclusion must however be considered as tentative because of the small size of the muscle 135d sample.

In 3 out of the 4 cases where motoneuron 30 innervates the correct muscles 135d and 135e, these muscles are also innervated by their correct excitatory motoneuron, D₆. This simultaneous foreign and correct innervation has been demonstrated at the level of the single muscle fibre.

In only one instance has motoneuron 30 been found to innervate a muscle other than a coxal depressor; this exception was a 135d flexor muscle (see section 4, 3, 3, 1).

data that motoneuron 30 takes up to 43 days to establish a functional connection with coxal depressor muscles and that, ultimately, it reinnervates these muscles in almost all animals.

B. REINNERVATION OF INDIVIDUAL COXAL DEPRESSOR MUSCLES

The connections of motoneuron 30 to individual coxal depressor muscles were assayed at various post-operative times (table 7). More data were obtained for muscle 137 than for other coxal depressor muscles as this muscle is the most accessible when the cockroach is set up for electrophysiological recording.

Motoneuron 30 innervates its appropriate target muscles in a high percentage of animals: muscle 137 innervated in 25/39 animals assayed (64%); 136 in 3/3 animals assayed; 135d[/] in 5/5 animals assayed; 135e[/] in 4/4 animals assayed. However, motoneuron 30 also innervates the foreign coxal depressor muscle 135d (at 32 and 53 days post crush) in 2 out of 3 animals assayed and the foreign muscle 135e (at 32 and 47 days) in 2 out of 3 animals assayed.

These figures suggest that motoneuron 30 makes inappropriate connections as frequently as correct connections since correct muscles 137, 136, 135d[/] and 135e[/] are innervated in 36 out of 51 cases (71%) while foreign muscles 135d, 135e are innervated in 4 out of 6 cases (67%). This conclusion must however be considered as tentative because of the small size of the muscle 135d, e sample.

In 3 out of the 4 cases where motoneuron 30 innervates the incorrect muscles 135d and 135e, these muscles are also innervated by their correct excitatory motoneuron, D_S. This simultaneous foreign and correct innervation has been demonstrated at the level of the single muscle fibre.

In only one instance has motoneuron 30 been found to innervate a muscle other than a coxal depressor: this exception was a tibial flexor muscle (see section 4.3.5.1).

TABLE 7: Reinnervation of individual coxal depressor muscles by motoneuron 30 at various times following nerve 5 crush

Assays: +; correlation of motoneuron 30 spikes with junction potentials from coxal depressor muscle or twitching of this muscle upon firing motoneuron 30.

-; no twitching of coxal depressor muscles when motoneuron 30 fired or no correspondence of junction potentials in muscle with motoneuron 30 spikes. (Only the latter assay was used for muscles 135d, e).

Note: Animals in which there was no morphological regeneration of nerve 5 are excluded from these figures.

Time
(days after crushing Nerve 5)

No. of animals

Muscle

	137		136		135d'		135e'		135d		135e	
	+	-	+	-	+	-	+	-	+	-	+	-
17-20	1	3	0	0	0	0	0	0	0	0	0	0
21-28	2	6	0	0	0	0	0	0	0	0	0	0
29-49	13	5	3	0	2	0	2	0	1	1	2	0
50+	9	0	0	0	3	0	2	0	1	0	0	1
Total	25	14	3	0	5	0	4	0	2	1	2	1

4.3.3.2 INNERVATION OF COXAL DEPRESSOR MUSCLES BY MOTONEURON D_S

Innervation of muscles by motoneuron D_S in regenerate animals was assayed, not by noting correspondence between junction potentials recorded from muscles and spikes recorded intracellularly from the soma of motoneuron D_S, but by looking for correspondence between muscle junction potentials and D_S spikes recorded extracellularly from nerve 5. D_S can often be identified unambiguously from extracellular nerve 5 recordings because it is the only large, tonically active unit in this nerve (see chapter 2). While this means of identification is unambiguous in normal animals it is possible that large tonic units recorded from nerve 5 in regenerates represent large axons which are phasic in normal animals but whose firing properties have been modified by the nerve crush operation to a tonic pattern. This possibility has not been excluded in the present study but it seems unlikely in view of the fact that D_S, as identified from nerve 5 recordings in regenerates, always innervates muscles which D_S innervates in normal animals.

A. INNERVATION OF COXAL DEPRESSOR MUSCLES AS A GROUP

Using the above assay D_S was found to have formed functional connections with at least one coxal depressor muscle in 35 out of 42 animals (83%) examined (table 8). It is difficult to demonstrate that motoneuron D_S does not innervate any coxal depressor muscles since this requires recording from all such muscles and looking for absence of correlation between muscle junction potentials and D_S spikes recorded from nerve 5. In the 7 animals where innervation by D_S was not found, the above criterion for negative innervation by D_S was applied to one or two muscles (most commonly 137) but in no case were all coxal depressor muscles tested. Thus, the estimate of 83% successful regeneration by D_S calculated from the present figures may be an underestimate.

Functional connections between D_S and coxal depressor muscles

TABLE 8: Innervation of at least one coxal depressor muscle by motoneuron D₅ at various times after crushing nerve 5.

Assays: +; correlation of junction potentials in at least one coxal depressor muscle with D₅ spikes.

-; (i) no correlation of junction potentials in at least one coxal depressor muscle with D₅ spikes; or

(ii) no activity in any coxal depressor muscles examined; or

(iii) no morphological regeneration of nerve 5.

Time
(days after
crushing nerve 5)

No. of animals

+ - (⁺/_{total})%

<u>REINNERVATION</u>			
17-19	* 1	1	50
20-28	7	2	78
29-43	13	1	93
44-227	14	3	82
Total	35	7	83

* This animal was assayed at 17 days post crush.

In 13 of the 22 animals in which D_2 misinnervated muscle 137 this muscle is also innervated by motoneuron 30; in three of these 22 animals 137 is not innervated by motoneuron 30 while in the other 8 cases it could not be determined whether motoneuron 30 innervates muscle 137 or not. As in the case for misinnervation of muscles 135 and 136, simultaneous forelimb/correl innervation has been demonstrated at the level of the single muscle fibre.

4.3.3.3 INNERVATION OF COXAL DEPRESSOR MUSCLES BY MOTONEURONS 30, 41 AND 42 (INHIBITORS)

Innervation of at least one coxal depressor muscle by inhibitory motoneurons, as assessed by the presence of hyperpolarizing junctions

were first detected at 17 days post crush. The percentage of animals in which D_S regenerates successfully has reached 78% by 20-28 days post crush. This figure is comparable to that found for the period 44 to 227 days (83%) suggesting that motoneuron D_S regenerates to the coxal depressor muscles within 28 days post crush.

B. REINNERVATION OF INDIVIDUAL COXAL DEPRESSOR MUSCLES

Connections of D_S to individual coxal depressor muscles were determined at various post operative times. Motoneuron D_S innervates its appropriate target muscles 135d, d', e, e' in every animal tested in the period 17 to 227 days (12, 8, 16 and 18 examples respectively). D_S also innervates the two foreign depressor muscles, 136 (in the one animal examined) and 137 (in 21 out of 25 animals examined), in the period 17-227 days post crush (table 9). In the period 20 to 52 days post crush, D_S fails to innervate muscle 137 in one out of 17 animals examined (94% reinnervation). However, from 53 to 227 days post crush, the successful innervation rate for this muscle drops to 63% (5 out of 8 animals), although D_S innervates 137 as late as 200 days post crush in one animal. These results suggest that, in the interval 17 to 52 days, D_S innervates foreign muscles as frequently as correct muscles (136, 137 innervated in 17 out of 18 cases [95%], muscles 135d', e', d, e innervated in 39 out of 39 cases [100%]).

In 13 of the 22 animals in which D_S misinnervates muscle 137 this muscle is also innervated by motoneuron 30; in three of these 22 animals 137 is not innervated by motoneuron 30 while in the other 6 cases it could not be determined whether motoneuron 30 innervates muscle 137 or not. As is the case for misinnervation of muscles 135d and e, simultaneous foreign/correct innervation has been demonstrated at the level of the single muscle fibre.

4.3.3.3 INNERVATION OF COXAL DEPRESSOR MUSCLES BY MOTONEURONS 40, 41 AND 44 (INHIBITORS)

Innervation of at least one coxal depressor muscle by inhibitory motoneurons, as assayed by the presence of hyperpolarizing junction

TABLE 9: Innervation of individual coxal depressor muscles by
motoneuron D₈ at various times after crushing nerve 5.

Assay: +; correlation of junction potentials in this muscle
with D₈ spikes.

-; no correlation between junction potentials recorded
in this muscle and D₈ spikes.

Time
(days after N5 crush)

No. of animals

	Muscle											
	137		136		135d/		135e/		135d		135e	
	+	-	+	-	+	-	+	-	+	-	+	-
17-19	0	0	0	0	1	0	0	0	0	0	0	0
20-28	0	1	0	0	2	0	3	0	3	0	3	0
29-43	12	0	1	0	5	0	2	0	7	0	4	0
44-52	4	0	0	0	1	0	2	0	2	0	4	0
53-227	5	3	0	0	3	0	1	0	4	0	7	0
Total	21	4	1	0	12	0	8	0	16	0	18	0

potentials in these muscles, was found in 9 out of 22 (41%) operated animals examined (table 10). In no case were such potentials recorded from muscles other than 135d and 135e. Table 11 shows the frequency with which IJP's were recorded from muscles 135d and 135e. While these figures suggest that inhibitory motoneurons regenerate far less readily than either motoneuron 30 or D_S, the assay used may underestimate the success of inhibitory motoneuron regeneration. Firstly, it is possible that the resting potential of muscles in regenerate animals is near the reversal potential for inhibitory junction potentials (IJP's); the IJP's in such muscles would be very small or not present. Secondly, inhibitory motoneurons may fire infrequently in regenerate animals.

The connections of the anatomically identified inhibitory motoneurons 40, 41 and 44 to the coxal depressor muscles were assayed by recording simultaneously from the depressor muscles and from the somata of these motoneurons. Positive innervation was indicated by correlation between muscle junction potentials and spikes in these motoneurons while negative innervation was indicated by absence of correlation between motoneuron spikes and junction potentials in the muscle fibres sampled.

The latter assay is open to the criticism that muscle fibres which were not sampled may have been innervated by inhibitory motoneurons. (Indeed in normal animals inhibitory motoneurons 40 and 41 do not innervate all fibres of muscles 135d, e; see fig. 10).

Reinnervation of an appropriate target muscle 135d by motoneuron 40 has been demonstrated in two animals at 49 and 193 days post crush (table 12). In another animal at 29 days post crush, no correlation was found between JP's recorded at various points on muscle 135d and motoneuron 40 spikes. The same finding has been made for muscle 135e, in single animals at 29 and 92 days post crush (table 12). Positive reinnervation of muscle 135d by motoneuron 41 has been demonstrated in one animal at 41 days post crush (table 12). In one animal, at 92 days post crush, no correlation was observed

TABLE 10: Innervation of at least one coxal depressor muscle
by inhibitory motoneurons at various times
following nerve 5 crush.

Assay: +; Hyperpolarizing JP's (assumed to result from activity in inhibitory motoneurons) recorded from these muscles.

-; No hyperpolarizing JP's recorded from either muscle 135d or 135e.

Time
(days after
N5 crush)

No. of animals

	+	-
17-19	0	1
20-28	1	3
29-43	3	4
44-227	5	5
Total	9	13

TABLE 11: Innervation of muscles 135d, e by inhibitory
motoneurons at various times after N5 crush.

Assay: +; Hyperpolarizing JP's recorded from this
muscle.

Time
(days after
N5 crush)

No. of animals

	Muscle			
	135d		135e	
	+	No. of animals in which this muscle recorded from	+	No. of animals in which this muscle recorded from
22-30	2		1	
31-49	3		1	
50-195	1		1	
Total	6	19	3	20

TABLE 12: Innervation of coxal depressor muscles 135d, 135e
by inhibitory motoneurons 40, 41 and 44 at various
times after crushing nerve 5.

+ Each entry in this column represents one
experimented animal.

Assays: +) see text.
 -)

+ Time
(days after
N5 crush)

	40 (D ₁)		41 (D ₃)		44 (D ₂)	
	Muscle		Muscle		Muscle	
	135d	135e	135d	135e	135d	135e
18					-	-
22					+	-
23						-
29	-	-				
30		-				
41			+			
41						-
49	+					
92		-	-			
193		+ (medial region)				
		- (lateral region)				

4.3.3.4 INNERVATION OF COXAL DEPRESSORS BY FOREIGN MOTONEURONS

In 21 of the 25 animals in which the coxal depressor muscles were functionally innervated, excitatory junction potentials (EJPs) were recorded from the depressor muscles which correlated with spike activity in either motoneuron 20 or 4₂ (Table 13). Since motoneurons 20 and 4₂ are the only excitatory motoneurons which innervate the coxal depressor muscles in normal animals, these EJPs must be produced by activity in "foreign" motoneurons (i.e.,

between muscle 135d inhibitory junction potentials and motoneuron 41 spikes. Motoneuron 44 reinnervated muscle 135d in one animal examined at 22 days post crush. Absence of correlation between muscle JP's and motoneuron 44 spikes was found in muscle 135d at 18 days post crush and in muscle 135e at 18, 22, 23 and 41 days post crush (table 12).

Clearly, then, the inhibitory motoneurons 40, 41 and 44 are capable of reinnervating their appropriate muscles. It would appear however that reinnervation of original muscle territories (section 2.3.2.3) is often unsuccessful. The latter conclusion must remain tentative, as in no case was the whole area of the muscle sampled: it is possible that "negative" recordings were made from points on muscles 135d and e which the inhibitors do not innervate in normal animals.

Since the inhibitory motoneurons 40, 41 and 44 appear to innervate particular regions of muscles 135d, e in normal animals (fig.10), it is of interest to consider whether these intramuscular patterns of innervation are re-established after regeneration. This question cannot be answered in most cases of positive reinnervation found here, as recordings were generally only made from limited regions of muscles 135d, e. However, in the animal at 193 days in which motoneuron 40 was found to have reinnervated muscle 135e, the medial part of this muscle was innervated while the lateral part was not, as is sometimes the case in normal animals.

4.3.3.4 INNERVATION OF COXAL DEPRESSORS BY FOREIGN MOTONEURONS

In 21 of the 45 animals in which the coxal depressor muscles were functionally innervated, excitatory junction potentials were recorded from the depressors during reflexive activity which did not correlate with spike activity in either motoneuron 30 or D₅ (table 13). Since motoneurons 30 and D₅ are the only excitatory motoneurons which innervate the coxal depressor muscles in normal animals, these extra EJP's must be produced by activity in "foreign" motoneurons (i.e.

TABLE 13: Innervation of at least one coxal depressor muscle
by "foreign" motoneurons at various times after
crushing nerve 5.

Assays: +; junction potentials recorded from at least one coxal depressor muscle which do not correlate with spike activity in motoneurons which normally innervate those muscles.

* "Examined", as used here, means that recordings were made from at least one coxal depressor muscle. In general, however, not all coxal depressor muscles were examined. It is possible that more animals would have been scored as "positive" if the coxal depressors have been more exhaustively tested.

Time
(days after
N5 crush)

No. of Animals

		Total No. of Animals "examined"*
17-28	2	9
29-43	11	15
44-52	3	7
53-218	5	14
Total	21	45

of four animals (25%); from muscle 135d in 5 out of 14 animals (35%); muscle 135d' in 5 out of 14 (35%) animals; from muscle 135e in 2 out of 21 animals (29%); and from muscle 135e' in 2 out of 14 animals (14%). (see table 14). These figures suggest that, with the exception of muscle 135e', all of the coxal depressor muscles are approximately equally readily innervated by foreign motoneurons.

In 24 out of 24 cases (100%) where a coxal depressor muscle is innervated by a foreign neuron, this muscle is also innervated by a correct neuron (motoneuron 30 and/or H_2). In 3 cases the muscle did not appear to be innervated by a correct neuron while in the other 21 cases it was unclear whether the muscle was also innervated by a correct neuron. Simultaneous foreign/correct innervation has been demonstrated at the level of a single muscle before (Fig. 19).

Foreign EJPs were found to be small in size (less than 1 mV). No more than three distinct sizes of foreign EJPs could be recorded from a muscle in any individual during reflexive activity. Fig. 30

neurons which do not normally innervate the coxal depressor muscles). Such foreign EJP's are recorded as early as 26 days post crush and as late as 218 days post crush.

The percentage of animals in which the coxal depressors are innervated by foreign motoneurons is higher at 29-43 days (11 out of 15 animals; 73%) than at 17-28 days (2 out of 9 animals; 22%), suggesting that foreign motoneurons may take up to 28 days to establish functional connections with coxal depressor muscles. The same percentage is lower at 44-52 days (3 out of 7 animals; 43%) than at 29-43 days and lower still at 53-218 days (5 out of 14 animals; 36%), suggesting that these foreign motoneurons are gradually eliminated after 43 days. These conclusions are tentative as the number of animals in which foreign motoneurons innervate coxal depressor muscles may be higher than actually recorded (see legend; table 13).

Foreign EJP's have been recorded from all of the coxal depressor muscles: from muscle 137 in 13 out of 39 (33%) animals in which JP's have been recorded from muscle 137; muscle 136 in one out of four animals (25%); from muscle 135d in 6 out of 19 animals (32%); muscle 135d' in 5 out of 14 (36%) animals; from muscle 135e in 6 out of 21 animals (29%); and from muscle 135e' in 2 out of 16 animals (13%), (see table 14). These figures suggest that, with the exception of muscle 135e', all of the coxal depressor muscles are approximately equally readily innervated by foreign motoneurons.

In 22 out of 34 cases (65%) where a coxal depressor muscle is innervated by a foreign neuron, this muscle is also innervated by a correct neuron (motoneuron 30 and/or D₅). In 4 cases the muscle did not appear to be innervated by a correct neuron while in the other 9 cases it was unclear whether the muscle was also innervated by a correct neuron. Simultaneous foreign/correct innervation has been demonstrated at the level of a single muscle fibre (fig. 19).

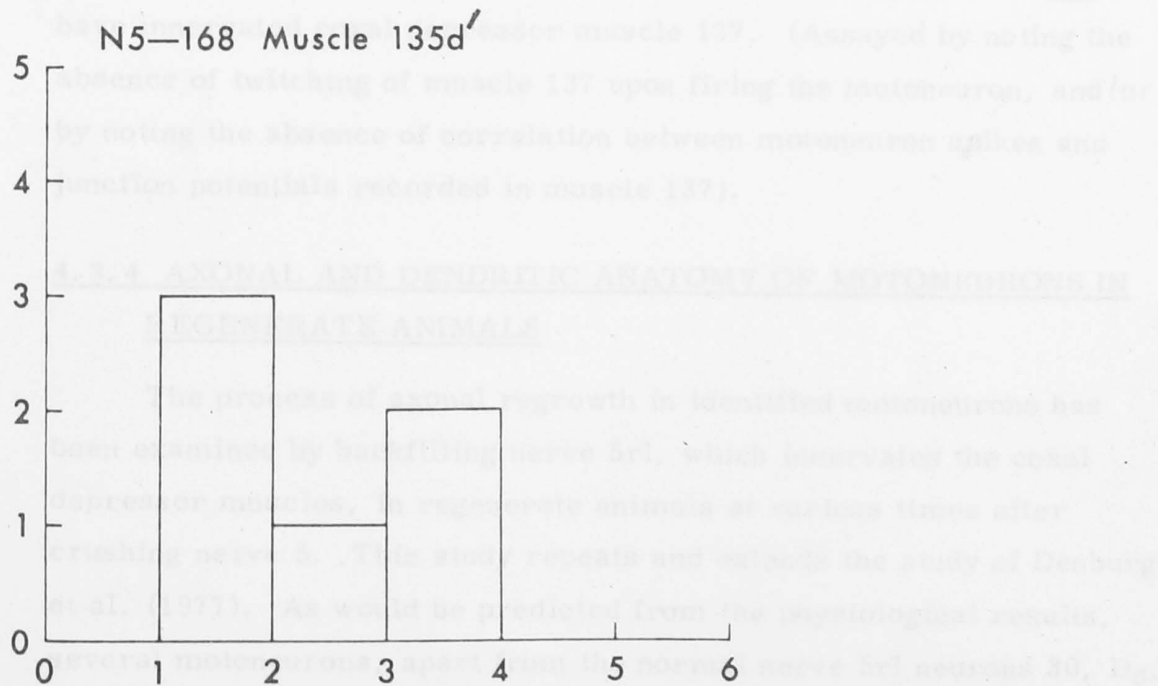
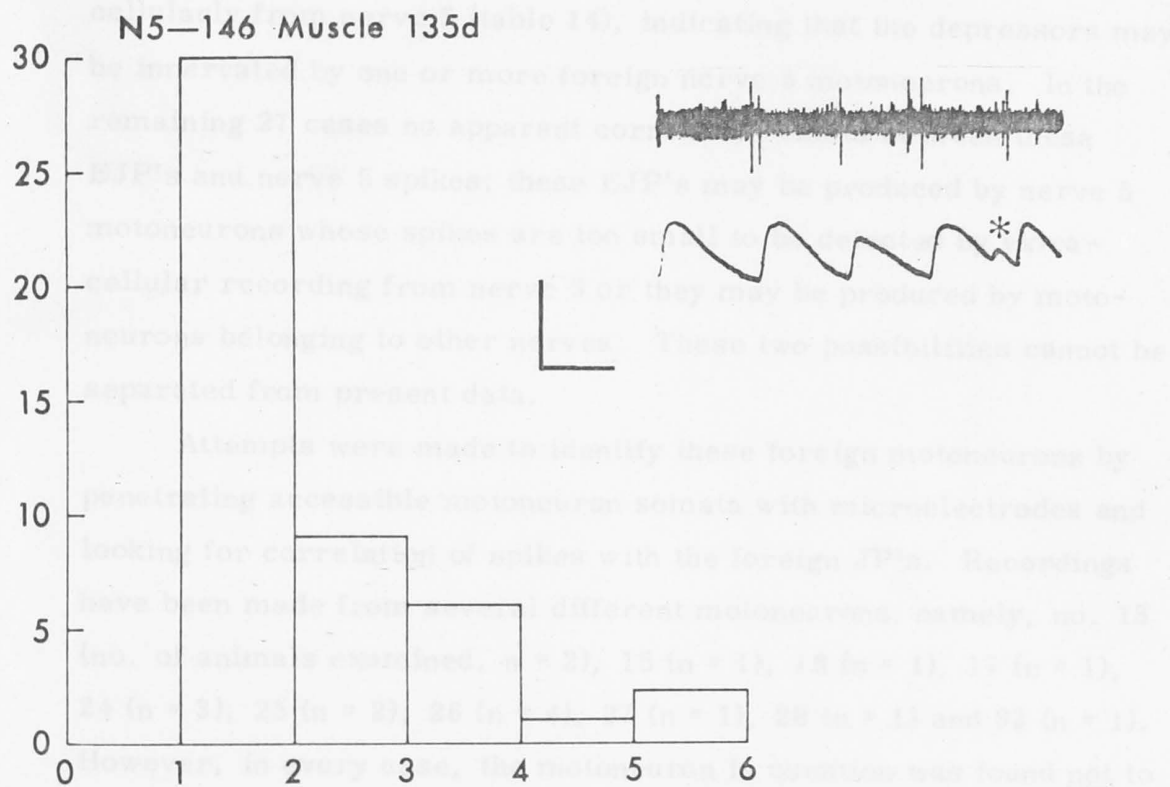
Foreign EJP's were found to be small in size (less than 1 mV). No more than three distinct sizes of foreign EJP's could be recorded from a muscle in any individual during reflexive activity. Fig. 20

Fig. 19 (insert)

Extracellular recording from nerve 5 (top trace) and intracellular recording from muscle 135d (bottom trace) in an animal (N5 - 146), 92 days after crushing nerve 5. This record demonstrates simultaneous innervation of a single muscle fibre by motoneuron D₅ (large EJP's correlated with large nerve 5 spikes) and by a foreign motoneuron (small EJP, asterisk), vertical bar 250 μ V (top trace), 2.5 mV (bottom trace), horizontal bar 10 msec.

Fig. 20

Amplitude distribution of foreign EJP's recorded from muscles 135d (animal N5-146) and 135d' (animal N5 - 168). Each histogram is derived from measurements of foreign EJP size in a single muscle fibre. Horizontal axis; size of foreign EJP in arbitrary units. Vertical axis; number of EJP's recorded which fall in this size class. Animal N5 - 146, 92 days post crush and animal N5 - 168, 39 days post crush.



shows the amplitude distribution of the two most complex patterns of foreign EJP activity which have been recorded. In 6 out of 33 cases foreign EJP's appeared to correlate with spikes recorded extracellularly from nerve 5 (table 14), indicating that the depressors may be innervated by one or more foreign nerve 5 motoneurons. In the remaining 27 cases no apparent correlation exists between these EJP's and nerve 5 spikes; these EJP's may be produced by nerve 5 motoneurons whose spikes are too small to be detected by extracellular recording from nerve 5 or they may be produced by motoneurons belonging to other nerves. These two possibilities cannot be separated from present data.

Attempts were made to identify these foreign motoneurons by penetrating accessible motoneuron somata with microelectrodes and looking for correlation of spikes with the foreign JP's. Recordings have been made from several different motoneurons; namely, no. 13 (no. of animals examined, $n = 2$), 15 ($n = 1$), 16 ($n = 1$), 17 ($n = 1$), 24 ($n = 3$), 25 ($n = 2$), 26 ($n = 4$), 27 ($n = 1$), 28 ($n = 1$) and 32 ($n = 1$). However, in every case, the motoneuron in question was found not to have innervated coxal depressor muscle 137. (Assayed by noting the absence of twitching of muscle 137 upon firing the motoneuron, and/or by noting the absence of correlation between motoneuron spikes and junction potentials recorded in muscle 137).

4.3.4 AXONAL AND DENDRITIC ANATOMY OF MOTONEURONS IN REGENERATE ANIMALS

The process of axonal regrowth in identified motoneurons has been examined by backfilling nerve 5rl, which innervates the coxal depressor muscles, in regenerate animals at various times after crushing nerve 5. This study repeats and extends the study of Denburg et al. (1977). As would be predicted from the physiological results, several motoneurons, apart from the normal nerve 5rl neurons 30, D₅, 40, 41 and 44, have been found to send axons down nerve 5rl. These "foreign" motoneurons come exclusively from the group of motoneurons

TABLE 14: Innervation of individual coxal depressor muscles
by "foreign" motoneurons (all post-operative
stages combined in this table).

Muscle	No. of animals in which foreign JP's recorded		No. of animals recorded from
	JP's correlated with N5 spikes	JP's uncorrelated with N5 spikes	
137	2	11	39
136	0	1	4
135d/	1	4	14
135e/	1	1	16
135d	1	5	19
135e	1	5	21

which send axons down nerve 5 in normal animals (see fig. 7). In any individual animal only a fraction of the total population of 28 nerve 5 motoneurons (figure determined from cobalt backfill studies in normal animals p. 27) send axons down nerve 5rl. At 21-56 days post crush, an average of 9.8 "foreign" motoneurons are filled through nerve 5rl in regenerates (table 15). There is a high degree of variability in the number of cells filled. It is unclear whether this variability is an artefact of the cobalt backfill technique or whether it represents real variability in the number of foreign axons present in nerve 5rl. The individual cells in this population of foreign motoneurons vary from animal to animal. The pattern is not random: some motoneurons send axons down 5rl in regenerates more frequently than others (fig. 21). In no case has a foreign motoneuron been found to send axons down nerve 5rl in more than 80% of animals examined. In contrast, four of the five normal nerve 5rl motoneurons D_S, 30, 40 and 44 are filled in 100% of "successful" preparations (i. e. preparations in which at least one cell body is filled). Motoneuron 41, which fills inconsistently in normal animals, is filled in only a low percentage of regenerate animals (ca. 10%).

There is evidence that "foreign" axons are withdrawn from nerve 5rl at late stages of regeneration: at 21 to 56 days post crush an average of 9.8 foreign motoneurons are filled through 5rl while at 64 to 222 days post crush this figure falls to 3.4 cells (table 15).

The dendritic geometry of identified motoneurons 30 and D_S in regenerate animals appears very similar to that in normal, unoperated animals, at least at the level of major dendritic branches (c.f. figs. 22 and 11a for motoneuron 30, figs. 23 and 12 for D_S; 10 cells examined for D_S, 3 for motoneuron 30). However, there are marked differences in axonal geometry between control and regenerate animals. The axons of motoneurons 30 and D_S in regenerate animals are often much thinner than in controls. Whereas axons of motoneurons 30 and D_S in normal animals show no signs of bifurcation in the region from the base of nerve 5 to branch 5rl, the same neurons in regenerate animals are always

TABLE 15: Number of incorrect cell bodies filled through
nerve 5rl in regenerate animals at various
times after crushing nerve 5.

Time
(days after
nerve 5 crush)

Mean No. of incorrect cell bodies filled

21-56

9.8 ± 4.9 (n = 9)

64-222

3.4 ± 3.3 (n = 5)

Fig. 21

Cell map of mesothoracic ganglion (from Young, 1973)
showing frequency with which identified neuron somata
fill through nerve 5rl in regenerate animals.

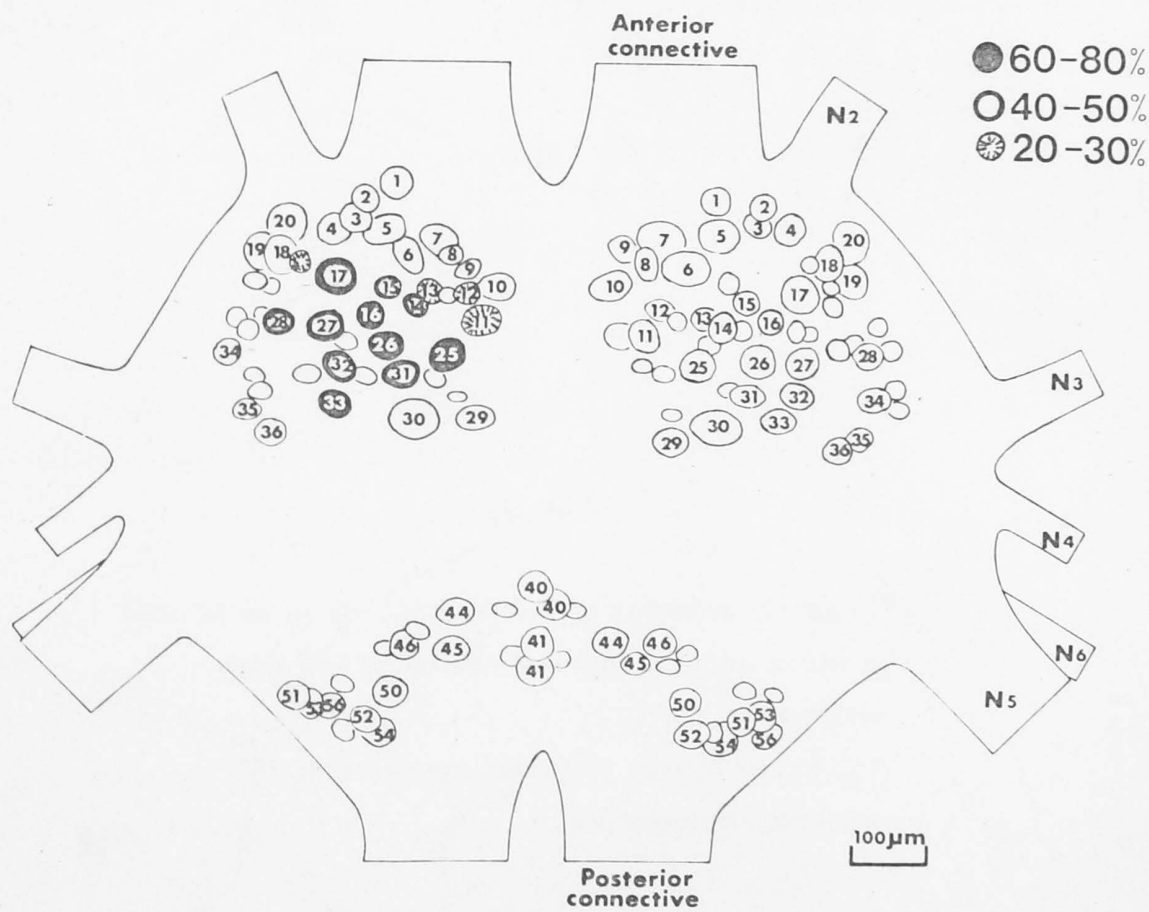


Fig. 22

Dendritic anatomy of motoneuron 30 in an animal in which nerve 5 had been crushed 22 days previously.

N5, nerve 5; AC, anterior connective; PC, posterior connective.

Fig. 23

Dendritic anatomy of motoneuron D₅ in an animal in which nerve 5 had been crushed 190 days previously.

N5, nerve 5; AC, anterior connective; PC, posterior connective.

branched distal to the crush site. The pattern of branching varies between individuals: in some cases branching is profuse and widespread, although directed distally, (fig. 24) while in other cases only two to three branches are apparent and these follow approximately the same course to nerve 5rl (fig. 25). The former pattern is more common at early stages but may also be seen at late stages (fig. 26). Branching occurs immediately distal to the crush site and in many cases from a point close to nerve branch 5rl. Whereas, in early stages, some branches appear to terminate before they reach nerve 5rl, at later stages, all branches appear to enter nerve 5rl (fig. 24, 25). As is the case in normal animals, axons of motoneuron 30 and D₅ often follow approximately the same course to nerve 5rl.

4.3.5 INNERVATION OF OTHER LEG MUSCLES

4.3.5.1 INNERVATION OF TIBIAL AND TARSAL FLEXOR MUSCLES

The nerve 5 crush operation, as well as denervating coxal depressor muscles, denervates tibial and tarsal flexor muscles which are located in the femur and tibia, respectively. Reinnervation of these muscles, as assayed by tibial or tarsal flexion on stimulating nerve 5 or individual motoneurons, occurred in 19 out of 23 animals (83%) examined, in the case of tibial flexor muscles, and in 11 out of 15 animals (73%), in the case of tarsal flexor muscles (table 16). These figures represent minimum successful reinnervation percentages as the negative innervation assay may have missed innervation of femoral/tibial muscles by "slow" motoneurons. Successful reinnervation of these muscles is first observed at 18 days post crush. The percentage of successful regenerates is higher at 29-43 days than at 18-28 days, and at 44-222 days tibial and/or tarsal flexor muscles had been reinnervated in all animals tested.

Innervation of tibial/tarsal flexor muscles by identified motoneurons was assayed by looking for tibial/tarsal flexion upon stimulating such motoneurons with intracellular depolarizing current pulses. Tibial flexor muscles were found to be innervated by

Fig. 24

Pattern of axonal branching in motoneuron 30 in an animal examined 47 days after crushing nerve 5.

This diagram was drawn from a preparation in which motoneuron 30 was injected with HRP through the soma.

N5, base of nerve 5; C, crush site; n5rl, nerve 5rl.

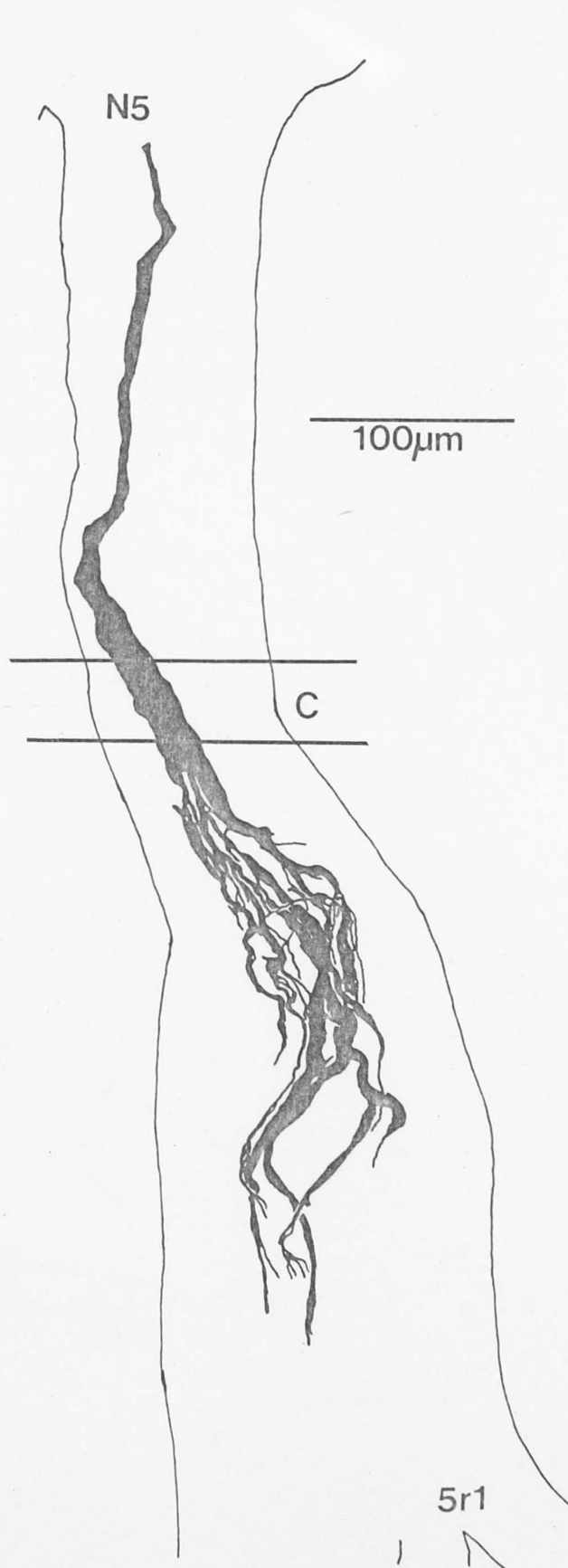


Fig. 25

Pattern of axonal branching in motoneuron D₅ in an animal examined 56 days after crushing nerve 5.

This diagram was drawn from a preparation in which nerve 5rl was backfilled with cobalt chloride.

N5, base of nerve 5; C, crush site; n5rl, nerve 5rl.

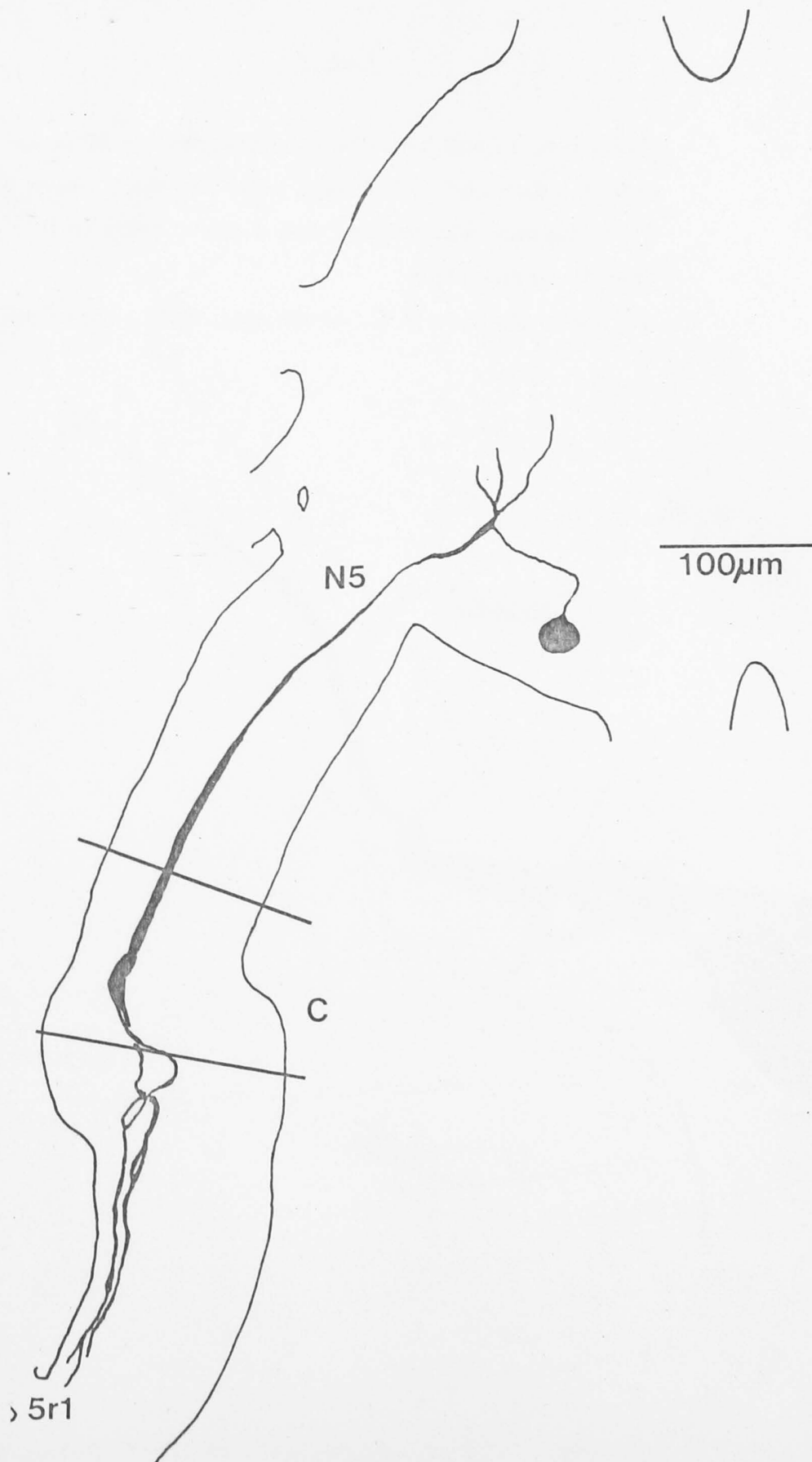


Fig. 26

Pattern of axonal branching in motoneuron 30 in an animal examined at 210 days after crushing nerve 5. This diagram was drawn from a nerve 5rl cobalt backfill preparation.

N5, base of nerve 5; C, crush site; N5rl, nerve 5rl.

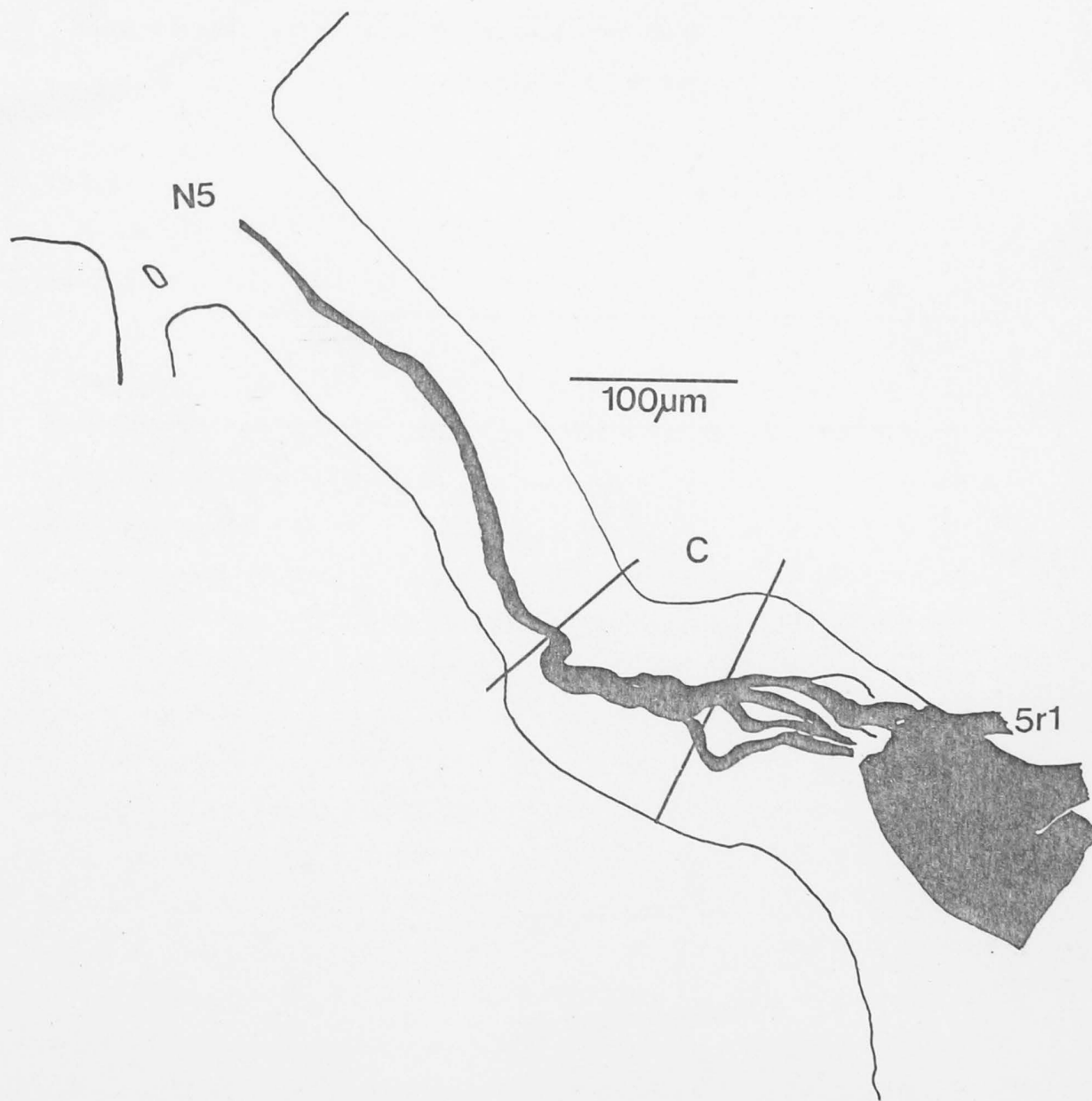


TABLE 16: Innervation of tibial and tarsal flexor muscles
by nerve 5 motoneurons at various times after
crushing nerve 5.

Assay: +; twitching of tibial or tarsal flexors when N5
 or N5 motoneurons stimulated.

 -; no twitching of tibial or tarsal flexors when
 nerve 5 stimulated.

Time (days p.o.)	No. of animals			
	Muscle			
	Tibial Flexors		Tarsal Flexors	
	+	-	+	-
18-28	5	3	5	3
29-43	9	1	4	1
44-222	5	0	2	0
Total	19	4	11	4

Nerve 5 was found to have innervated tibial extensor muscles in 18 of 22 animals assayed 18-28 days post-crush (table 18). In one animal, the tibial extensor in this animal was no. 24 (table 18) innervated by noting tibial extension when this motoneuron was fired. In another animal, at 42 days post-crush, the tibial extensor muscle was shown to be innervated by a different identified motoneuron no. 27. (table 18).

Two identified motoneurons, nos. 27 and 13, innervate coxal levator muscles in regenerates at 80 and 33 days post-crush respectively (one animal in each case; table 18).

All of the above connections represent misinnervation since a) nerve 5 does not innervate either tibial extensor or coxal levator muscles in normal animals (chapter 25 of motoneurons 18, 24 and 27 send axons out nerve 5 in normal animals (see fig. 7).

These results, taken with those in table 17, show that some identified motoneurons may innervate different muscles in different animals. Specifically: motoneuron no. 13 innervates tibial flexors in two animals and femoral flexor muscles in one animal; no. 24 innervates, in different individuals, tibial flexor, tarsal flexor and tibial extensor muscles; no. 32 innervates tibial flexors and tarsal

motoneurons 13, 16, 24, 25, 26, 30 and 32 while tarsal flexors were innervated by motoneurons 24 and 32 (table 17). Innervation of a tibial flexor muscle by motoneuron 30 represents misinnervation as motoneuron 30 innervates coxal depressor muscles exclusively in unoperated animals (see chapter 2). This particular mistake is found at a late stage, namely 222 days post crush. In the remaining cases it could not be determined whether innervation of the tibial or tarsal flexor muscles by identified motoneurons represented correct or incorrect innervation as the connections of these motoneurons to leg muscles in normal animals are not known.

4.3.5.2 INNERVATION OF TIBIAL EXTENSOR AND COXAL LEVATOR MUSCLES

Nerve 5 was found to have innervated tibial extensor muscles in one animal assayed at 32 days post crush (assay: extension of tibia when nerve 5 was stimulated). One of the motoneurons innervating the tibial extensors in this animal was no. 24 (table 18) (assayed by noting tibial extension when this motoneuron was fired). In another animal, at 32 days post crush, the tibial extensor muscles have been shown to be innervated by a different identified motoneuron no. 27, (table 18).

Two identified motoneurons, nos. 27 and 13, innervate coxal levator muscles in regenerates at 30 and 22 days post crush respectively (one animal in each case; table 18).

All of the above connections represent misinnervation since:

- a) nerve 5 does not innervate either tibial extensor or coxal levator muscles in normal animals (chapter 2); b) motoneurons 13, 24 and 27 send axons out nerve 5 in normal animals (see fig. 7).

These results, taken with those in table 17, show that some identified motoneurons may innervate different muscles in different animals. Specifically: motoneuron no. 13 innervates tibial flexors in two animals and femoral flexor muscles in one animal; no. 24 innervates, in different individuals, tibial flexor, tarsal flexor and tibial extensor muscles; no. 32 innervates tibial flexors and tarsal

TABLE 17: Reinnervation of tibial/tarsal flexor muscles
by anatomically identified motoneurons.

Assay: +; twitching of these muscles when neuron
 fired.

* indicates that the motoneuron in question
was not identified unequivocally.

Motoneuron No.	Time (days p.o.)	Tibial Flexors	Tarsal Flexors
*13	23	+	
*13	30	+	
16	216	+ (143b?)	
*24	193	+ (143b?)	
24	216		+
25	200	+ (143b?)	
25	30	+	
26	34	+	
*26	39	+	
*26	41	+ (143a?)	
30	222	+ (143b?)	
32	34	+	
*32	23		+

TABLE 18: Misinnervation of coxal levator and tibial
extensor muscles by anatomically identified
motoneurons.

Assay: +; twitching of muscles when motoneuron
 fired.
 -; no twitching of muscle when motoneuron
 fired.

Motoneuron No.	Animal No.	Time p.o. (days)	Coxal Levator	Tibial Extensors
13	27	22	+	-
24	205	32	-	+
27	205	32	-	+
27	127	30	+	-

2.4. DISCUSSION

2.4.1. ABILITY OF NEURONS TO REGENERATE AND OF MUSCLES TO BE REINNERVATED

The percentage of animals examined in which a given motor neuron or motor nerve innervates at least one leg muscle gives an indication of the ability of that neuron or nerve to regenerate. This figure was, for the post-operative period from 17 to 334 days, 61% for nerve 5, 55% for motoneuron 22, 23% for motoneuron D₂ and 41%

flexors in different individuals; no. 27 innervates femoral flexor and tibial extensors in different animals.

Other motoneurons consistently innervate the same group of muscles in different individuals. Specifically: motoneuron 26 innervates tibial flexor muscles in three different animals (table 17); motoneuron no. 25 innervates tibial flexor muscles in both individuals assayed (table 17).

4.3.5.3 MISINNERVATION IN PRESENCE OF CORRECT TARGETS

As in the muscle excision study, several animals were found in which motoneurons innervated foreign muscles and as far as could be determined from the assay used, not their correct muscles. These cases include: a) misinnervation of tibial flexor muscles by motoneuron 30 (coxal depressors not innervated by motoneuron 30 in this animal); b) misinnervation of coxal levator muscles by motoneurons 13 and 27 (tibial, tarsal flexors not innervated by these motoneurons, table 18); and c) misinnervation of tibial extensor muscles by motoneurons 24 and 27 (tibial/tarsal flexor muscles not innervated by these motoneurons in these animals). (Table 18).

Again, both the explanation that their correct target muscles are not physically accessible to these neurons and the explanation that these neurons had not regrown to their correct muscles at the time the connections were assayed appear implausible.

4.4 DISCUSSION

4.4.1 ABILITY OF NEURONS TO REGENERATE AND OF MUSCLES TO BE REINNERVATED.

The percentage of animals examined in which a given motor neuron or motor nerve innervates at least one leg muscle gives an indication of the ability of that neuron or nerve to regenerate. This figure was, for the post-operative period from 17 to 228 days, 81% for nerve 5; 63% for motoneuron 30; 83% for motoneuron D₅ and 41%

for inhibitory motoneurons.

Using the cobalt backfilling technique, Denburg et al. (1977) have found that in 75% of cockroaches examined from 17 to 94 days after crushing nerve 5, axons have regenerated to a point just proximal to the trochanter. In addition they have shown that the axon of motoneuron 30 regenerates to nerve branch 5r1 in 67% of operated animals examined between 20 and 95 days post crush whereas this axon is present at this level in 100% of control (unoperated) animals. The corresponding figures for motoneuron D_S were 74% in operated animals and 92% in controls. Thus the estimates of regeneration capacity of nerve 5 and motoneurons D_f and D_S derived in the present study from physiological data are similar to those derived from morphological data by Denburg et al. (1977).

At least one of the coxal depressor muscles 137, 136, 135d, 135d', 135e, 135e', is innervated in 77% of animals examined from 12 to 228 days post crush. This result shows that the depressors are innervated as frequently as nerve 5 reinnervates leg muscles.

Functional reinnervation of muscles 137, 136, 135d, 135d', 135e and 135e' was found in 75%, 87%, 80%, 95%, 61% and 80%, respectively, of animals examined from 12 to 228 days post crush. These differences may reflect real differences in the accessibility of these muscles to innervation by regenerating motoneurons or they may merely result from differences in ability to record activity which is present in the muscle.

Tibial and tarsal flexor muscles were innervated in 83% and 73%, respectively, of animals tested, suggesting that these muscles are as accessible to regenerating motoneurons as the coxal depressors.

It was observed in several animals that muscles 136 and 137 showed signs of atrophy at early stages of regeneration or at late stages in muscles where no signs of reinnervation were detected. These results suggest that muscles 136 and 137 may degenerate in the absence of functional innervation. In comparison, Nüesch and Teutsch (1968) have found that muscle 178 (the metathoracic homologue of 136)

never reaches the size of muscles in normal adult animals if nerve 5 is cut in nymphal cockroaches, suggesting that innervation may be required for full growth as well as maintenance of cockroach muscles.

4.4.2 TIME REQUIRED FOR INNERVATION OF LEG MUSCLES BY REGENERATING MOTONEURONS

The percentage of animals which show innervation of coxal depressor muscles by nerve 5 increases from 63% at 12 to 28 days post crush to 83% at 29 to 43 days post crush and to 89% at 44 to 228 days post crush. Functional reinnervation of femoral and tibial muscles by regenerating nerve 5 motoneurons was first observed at 18 days post crush. The percentage of animals in which femoral flexor connections were found rose from 63% of those tested at 18 to 28 days post crush to 90% at 29 to 43 days and 100% at 44 to 222 days post crush. These figures suggest that: a) nerve 5 may take up to 29 days to re-establish functional connections with leg muscles after crushing, b) the time course of innervation of femoral muscles is similar to that for coxal muscles.

At 20-28 days motoneuron 30 had re-established functional connections with at least one coxal depressor muscle in 38% of animals tested; by 29-43 days this number had increased to 64%, then to 93% at 44-227 days. If these figures give a true indication of the percentage of successful regenerates at these times (see page 52), then it appears that up to 43 days may be required for innervation of the coxal depressor muscles by motoneuron 30. By comparison Denburg et al. (1977) found that, following nerve 5 crush, motoneuron 30 sends an axon down nerve branch 5r1 (which innervates the coxal depressors) in 58% of animals examined between 20 and 44 days post crush and in 75% of animals examined at 49 to 95 days post crush. Their results also suggest that in some animals, motoneuron 30 regenerates to the coxal depressors as late as 44 days post crush.

The present results suggest that D_S regenerates to the coxal depressor muscles within 28 days after nerve crush. Denburg et al.

(1977) find no change in the percentage of animals (75%) in which the axon of D_5 fills through nerve 5rl between 20-44 and 45-95 days after nerve 5 crush. This result is consistent with the present estimate for the time required for D_5 to regenerate to the depressors. Inhibitory motoneurons have been found to innervate coxal depressor muscles as early as 22 days after nerve 5 crush (in the case of motoneuron no. 44). However, insufficient data are available to estimate the maximum time required for regrowth of inhibitory neurons to the depressors.

"Foreign" motoneurons (see section 4.3.3.4) appear to require up to 43 days to innervate coxal depressor muscles since the percentage of animals in which "foreign" JP's are found in the depressors rises from 22% at 17-28 days to 73% at 29-43 days. Thereafter the percentage of misinnervation falls; see section 4.4.5.

4.4.3 PATTERNS OF AXONAL BRANCHING OF NERVE 5 MOTONEURONS

The data on the frequency with which identified motoneurons send axons down nerve 5rl point to the following conclusions: the normal nerve 5rl motoneurons D_5 , 30, 40 and 44 invariably send axons down nerve 5rl, while the other foreign nerve 5 motoneurons grow down this branch in only some animals. This result accords with the finding of Denburg et al. (1977), although the extent of foreign axon ingrowth into nerve 5rl appears to be greater in the present study (9.8 foreign neurons filled through 5rl in the period 22 to 56 days in the present study, 3.4 foreign cells filled in the period 20 to 44 days in the study of Denburg et al.). At least two explanations, which cannot be separated using present data, can be proposed to account for this difference: a) foreign neurons in the metathoracic ganglion, which Denburg et al. studied, grow down nerve 5rl less readily than in the mesothoracic ganglion; and b) the cobalt backfill technique used here fills a higher percentage of neurons which are present in nerve 5rl than the technique of Denburg et al.

The choice of which foreign neurons will send axons down nerve

5rl appears to be non-random: some identified neurons are filled through 5rl more frequently than others. Whether these differences result from non-specific factors (e.g. axons closest to 5rl favoured, smallest axons favoured) or to specific differences in the ability of foreign motoneurons to recognise and follow a path to branch 5rl is discussed in section 5.3.1.

"Correction" of these mistakes, i.e. elimination of foreign axons from branch 5rl, is inferred from the fall in number of incorrect neurons filled through nerve 5rl between the periods 21 to 35 days and 64 to 222 days post crush. Denburg et al. (1977) have noted a similar decline in the number of foreign neurons filled at later post-operative times (3.4 incorrect cells filled at 20 to 44 days, 0.33 cells filled at 45 to 95 days post crush in their study). The present study shows that correction is not perfect as foreign axons have been detected in nerve 5rl as late as 222 days post crush.

Because of the limitations of the cobalt backfill technique (see page 28) foreign branches present in nerve 5rl may have gone undetected in some cases. This fact may have the following consequences:

a) The degree of misdirection of foreign axons down nerve 5rl may be higher than actually reported, i.e. the real average no. of foreign neurons present in 5rl may be higher than the 9.8 cells found at 21-35 days post crush.

b) The conclusion that foreign axons do not invariably grow down nerve 5rl may not be valid since all nerve 5 motoneurons may grow down nerve 5rl in every animal but only a fraction of these may be detected. This possibility seems unlikely but even if it were true it would need to be explained why the correct nerve 5rl motoneurons are always detected in nerve 5rl by the same technique.

c) The observed differences in the frequency with which foreign motoneurons send axons down nerve 5rl may be artefactual, i.e. all foreign neurons may be present in the same percentage of animals but some neurons may be detected more frequently than others. This possibility cannot be excluded at present.

d) Correction of mistakes may not occur; rather foreign axons which are present in nerve 5rl may fill less frequently at later stages than at earlier stages. There exists no obvious reason why this should occur.

It may appear that to obtain an unambiguous estimate for the number of neurons sending axons down nerve 5rl, one only needs to cut sections of this nerve and count the number of axons. However, this method assumes that each neuron sends only one axon down nerve 5rl: this is clearly not the case in regenerate animals (see below).

Four conclusions can be drawn from the pattern of axonal branching seen in motoneurons 30 and D₅ distal to the crush site:

- a) axonal branching may be widespread or restricted but in every case the axon bifurcates at least once before reaching nerve 5rl;
- b) regenerating axons may take more than one path to nerve 5rl;
- c) axonal branches which fail to reach nerve 5rl may "die back" since such branches are often seen at early stages of regeneration but have not been seen at later stages;
- d) axons may branch either immediately distal to the crush point or close to nerve 5rl.

Widespread axonal branching immediately distal to the crush site has also been observed in regenerating leech sensory (Miyazaki et al., 1976) and interneurons (Fernandez and Fernandez, 1974; Carbonetto and Muller, 1977).

4.4.4 PATTERN OF INITIAL FUNCTIONAL CONNECTIONS

4.4.4.1 SPECIFIC vs. INCORRECT INNERVATION

A number of anatomically identified motoneurons have been shown, in this study, to be capable of re-establishing functional connections with their correct target muscles after axotomy: motoneuron 30 reinnervates muscles 136, 137, 135d¹, 135e¹, as has been previously demonstrated (Pearson and Bradley, 1972); the inhibitory motoneurons 40, 41 and 44 have all been shown, for the first time, to be capable of

reinnervating muscle 135d.

In addition, however, motoneurons have also been found to innervate inappropriate muscles after regenerating to an intact leg. For example, motoneuron 30 innervates coxal depressor muscles 135d, 135e and tibial flexor muscles; motoneuron D_S innervates muscles 136, 137; a number of unidentified motoneurons misinnervate coxal depressor muscles; motoneuron no. 27 misinnervates tibial extensor and coxal levator muscles; motoneuron no. 24 misinnervates tibial extensor muscles; and motoneuron no. 13 misinnervates coxal levator muscles.

Clearly, the option of innervating inappropriate muscles is open to at least some motoneurons regenerating to a full set of leg muscles. "Respecification" of muscle identities was advanced as a possible explanation for apparent misinnervation in the muscle excision study (page 41). This explanation seems unlikely in the present experiments as no muscles were removed from the leg and surgical interference, in general, was kept to a minimum.

We can therefore confidently reject the possibility that specific reinnervation results solely from the rule "motoneurons reinnervate only appropriate target muscles".

4.4.4.2 RESTRICTIONS ON INNERVATION

Granted that initial innervation does not take place on an exclusive "a motoneuron may only innervate its correct muscles" basis, are there any restrictions on functional synapse formation or is initial innervation a random process? If restrictions do occur we may consider whether they support the existence of specific rules for connectivity (see section 1.5).

Two candidate rules for synapse formation can be rejected on the basis of cases of misinnervation reported here. The finding that motoneuron 30, a "fast" motoneuron, can innervate the "slow" muscles 135d, e eliminates the rule, "fast" motoneurons may not innervate "slow" muscles. The result that motoneuron D_S, a "slow" motoneuron,

has been found to innervate the "fast" muscles 136, 137 eliminates the rule, "slow" motoneurons may not innervate "fast" muscles.

However, several findings in this study indicate that initial innervation is not random but takes place under certain restrictions. As in the muscle excision study the existence of two classes of restrictions is suggested by the patterns of innervation: general restrictions, which apply regardless of the individual identities of the motoneuron or muscle involved; and specific restrictions, which apply only to particular combinations of motoneurons and muscles.

A. GENERAL RESTRICTIONS

The restriction that a regenerating motoneuron may innervate only one muscle at a time, which was suggested from the muscle excision study, is not supported by the present study. In single animals, both motoneurons 30 and D₅ may innervate more than one muscle from the group 136, 137, 135d, d', e, e'. The fact that in the muscle excision study, regenerating motoneurons did tend to innervate only one muscle, may have been due to the fact that only one or two denervated muscles were available in this experimental situation.

The restriction that regenerating motoneurons may only innervate muscles which have been previously denervated does receive some qualified support. Innervation by regenerating motoneurons of muscles which are not innervated by nerve 5 in normal animals, was found in only four out of 61 animals examined physiologically (see table 17). The muscles involved were tibial extensors (normally innervated by nerve 3B) and coxal levators (normally innervated by nerve 6). These four exceptions indicate that this restriction is not absolute, but it certainly seems to apply to regenerating motoneurons in most cases. The possibility cannot be excluded that this behaviour of regenerating motoneurons is a consequence of a specific restriction: that is, regenerating nerve 5 motoneurons innervate muscles normally innervated by nerve 5, not because these are the only denervated

muscles, but because these motoneurons recognise and preferentially innervate nerve 5 muscles rather than, say, nerve 4 muscles.

A final general restriction, which was also suggested by the data from the muscle excision study, is that there is a limit to the number of motoneurons that a muscle can accommodate at any one time. This restriction is inferred from the observation that no more than three distinct sizes of "foreign" EJP's (i.e. EJP's which do not correlate with spike activity in appropriate neurons) were recorded from coxal depressor muscles. Subject to the reservations expressed on page 40, this result suggests that no more than five (three foreign plus up to two correct) out of a population of approximately 25 regenerating excitatory motoneurons in nerve 5 innervate an individual coxal depressor muscle. That many more than three foreign motoneurons regrow to the coxal depressor muscles is suggested by the observation that an average of 9.8 motoneurons send axons down nerve branch 5r1 to the coxal depressor muscles at early stages of regeneration (as inferred from cobalt backfill studies). Again this apparent general restriction on initial innervation may, in fact, represent a specific restriction: it is possible that only three motoneurons misinnervate individual coxal depressor muscles because only three specific nerve 5 motoneurons, other than the correct neurons, are "acceptable" to an individual coxal depressor muscle. If this were so we would expect an individual coxal depressor muscle to be misinnervated by the same three foreign motoneurons in different animals. It cannot be determined, from present results, whether this is so.

B. SPECIFIC RESTRICTIONS

Some results from the present study argue for the existence of specific restrictions on the initial formation of neuromuscular connections.

Firstly, IJP's have only been recorded from the coxal depressor muscles, 135d and e, which are the only coxal depressor muscles innervated by the inhibitory motoneurons D₁, D₂, D₃ in normal,

unoperated animals. This result would seem to suggest the rule: "inhibitory motoneurons may not innervate foreign muscles".

However, the fact that IJP's were not recorded from the other coxal depressor muscles does not necessarily indicate that the inhibitors do not innervate these muscles for the reasons discussed on page 56.

Secondly, motoneuron 30 has been found to innervate tibial or tarsal flexor muscles in only one out of 28 animals examined; in the other 27 cases coxal depressor muscles alone were innervated. One may argue from this result, for the existence of the rule: "motoneuron 30 may not innervate tibial or tarsal flexor muscles". However, several objections can be raised against this conclusion:

a) the assay used to establish negative innervation by motoneuron 30 is not perfect; functional innervation of "slow" tibial/tarsal muscles or of small numbers of fibres in any muscle may have gone undetected; b) it is not known whether motoneuron 30 commonly regrows beyond the coxa; if it does not, then we cannot draw any conclusions about the ability of motoneuron 30 to innervate tibial/tarsal flexor muscles; c) even if motoneuron 30 does regrow to the femur and beyond, the correct tibial/tarsal flexor neurons may precede it, in which case motoneuron 30 may be prevented from innervating these muscles because they are already innervated.

Thirdly, some nerve 5 motoneurons consistently fail to innervate the coxal depressor muscle 137, viz. motoneuron nos. 26, 24 do not innervate muscle 137 in 4 out of 4 and 3 out of 3 animals examined, respectively. Again these results do not necessarily support the rule "motoneurons 24 and 26 may not innervate coxal depressor muscle 137" for several reasons: a) innervation of small numbers of fibres of muscle 137 may have gone undetected by the method used to assay connections of these motoneurons; b) these motoneurons may not send axons down the nerve branch of 5r1 which innervates muscle 137; c) motoneuron 30 may reach muscle 137 before motoneurons 24 and 26, in which case the latter neurons may be denied the opportunity of innervating 137.

Data on the frequency with which identified motoneurons make connections with different muscles may suggest rules for connectivity. For example, it appears that motoneuron D_S initially innervates coxal depressor muscles randomly; there is no indication that the correct muscles 135d', e', d, e are "preferred" over the incorrect muscles 136, 137 (since the former muscles are innervated in 37 out of 37 cases, latter in 17 out of 18 cases at 20-52 days post crush).

Too few figures are available to comment on whether motoneuron 30 prefers to innervate its correct muscles 136, 137, 135d', e' over the foreign muscles 135d, e or whether other identified nerve 5 motoneurons e.g. 24, 25, 26, "prefer" some muscles over others. Further experiments may reveal trends of this type, however.

4.4.5 CORRECTION OF INITIAL MISINNERVATION

The suggestion that some of the "mistakes" (i. e. functional connections on inappropriate muscles) made by regenerating cockroach motoneurons at early stages of reinnervation may be corrected at later stages, receives some support from the present study. Firstly, motoneuron D_S misinnervates muscle 137 in 94% of animals examined between 20 and 52 days post crush but in only 62% of animals examined between 53 and 227 days. Secondly, "foreign" JP's (i. e. junction potentials which do not correlate with spike activity in appropriate motoneurons) are recorded from coxal depressor muscles in 73% of animals examined between 29 and 43 days post crush. This figure falls to 43% at 44-52 days and 36% at 53-218 days. These figures suggest that correction of misinnervation begins around 40 to 50 days post crush.

It is clear, however, that correction of "mistakes" (at least over the period examined) is not perfect. Inappropriate connections are found at late post operative times. For example, motoneuron 30 misinnervates tibial flexor muscles at 222 days post crush, motoneuron D_S misinnervates muscle 137 at 60, 68, 92 and 200 days post crush and

"foreign" motoneurons innervate coxal depressors at 92, 193, 195, 200 and 218 days post crush. While it is possible that these mistakes may be corrected at still later post operative times it would appear that reinnervation of muscles by motoneurons is not as accurate as innervation during development.

CHAPTER 3

GENERAL DISCUSSION

3.1 PLASTICITY OF INVERTEBRATE MOTOR NEURONS

Insertion of foreign muscles by regenerating motor nerves has been well documented by a wide range of vertebrates (e.g. mammals, 1866, 1875; fish, Sperry and Arora, 1965; arthropods, Calkins et al., 1973). The present study represents the first definitive report of functional innervation of a foreign muscle by a regenerating invertebrate motoneuron. Thus the rule "motoneurons may innervate only appropriate muscles", does not apply to all motoneurons in the cockroach at least. Studies of the type reported here need to be repeated in other invertebrates in order to assess whether any general

CHAPTER 5

conclusions can be drawn regarding the plasticity of invertebrate versus vertebrate GENERAL DISCUSSION

5.1 DECISION POINTS DURING NEURONAL DEVELOPMENT

A question of major interest in this study has been when crucial decision points or "gates" occur during neuronal development (see page 13). Answers to two questions may be expected to shed light on this problem:

- 1) Do all neurons growing to a particular target cell reach a given stage in development? and
- 2) Does a particular neuron reach a given stage in development when growing to all target cells?

5.2.1 GATES DURING AXON OUTGROWTH

Two results from the nerve 3 crush study suggest that gates may occur during axon outgrowth:

- 1) whereas motoneurons 30, 40, 43 and 44 invariably send axons down branch 5r1, other nerve 3 motoneurons grow down this branch in only some animals,
- 2) within the latter group of "foreign" nerve 3 motoneurons, some send axons down nerve 5r1 more frequently than others.

Support for axon outgrowth as an important decision-making stage

5.1 PLASTICITY OF INVERTEBRATE VERSUS VERTEBRATE MOTONEURONS

Innervation of foreign muscles by regenerating motor nerves has been well documented in a wide range of vertebrates (e.g. mammals, Hoh, 1975; fish, Sperry and Arora, 1965; amphibians, Cass et al., 1973). The present study represents the first definitive report of functional innervation of a foreign muscle by a regenerating invertebrate motoneuron. Thus the rule "motoneurons may innervate only appropriate muscles", does not apply to all motoneurons in the cockroach at least. Studies of the type reported here need to be repeated in other invertebrate species to assess whether any general conclusions can be drawn regarding the plasticity of invertebrate versus vertebrate motoneurons.

5.2 DECISION POINTS DURING NEURONAL DEVELOPMENT

A question of major interest in this study has been when crucial decision points or "gates" occur during neuronal development (see page 19). Answers to two questions may be expected to shed light on this problem:

- 1) Do all neurons growing to a particular target cell reach a given stage in development? and
- 2) Does a particular neuron reach a given stage in development when growing to all target cells?

5.2.1 GATES DURING AXON OUTGROWTH

Two results from the nerve 5 crush study suggest that gates may occur during axon outgrowth:

- 1) whereas motoneurons 30, D₅, 40 and 44 invariably send axons down branch 5r1, other nerve 5 motoneurons grow down this branch in only some animals,
- 2) within the latter group of "foreign" nerve 5 motoneurons, some send axons down nerve 5r1 more frequently than others.

Support for axon outgrowth as an important decision-making stage

comes from a variety of studies in both the vertebrates and invertebrates. The main line of evidence is that regenerating motor and sensory nerves do not grow out randomly but re-establish normal patterns of branching: Piatt (1957) finds that the pattern of motor nerve branching in a regenerated axolotl limb is similar to that observed in normal, adult limbs; Van Essen and Jansen (1976) have shown that regenerating identified motor and sensory neurons in the leech only send axons out their normal nerve roots; and in goldfish, regenerating fibres from dorsal and ventral halves of the retina selectively avoid one branch of the optic tract in favour of the branch they would normally occupy (Attardi and Sperry, 1963; Horder, 1974). (Constancy in the final pattern of nerve or axonal branching does not, in itself, provide evidence for decision-making during axon outgrowth; time course studies are required to exclude the possibility that axons branch randomly initially and that branches which do not terminate on appropriate targets regress.) A second line of evidence for decision-points during axon outgrowth is that surgical manipulations which would be expected to disrupt regrowth of neurons down their normal pathways may lead to innervation of foreign targets by these neurons: regenerating leech sensory neurons tend to innervate foreign skin areas more frequently after nerve cutting than after nerve crushing (Van Essen and Jansen, 1976); some optic nerve fibres which are forced to grow down a foreign nerve root innervate inappropriate regions of the brain (Hibbard, 1967). Such operations do not necessarily lead to misinnervation, however: in many cases neurons may terminate at appropriate post-synaptic sites albeit growing down abnormal pathways (see section 5.3.1).

5.2.2 GATES PRIOR TO FUNCTIONAL SYNAPSE FORMATION

Several results from the muscle excision and nerve 5 crush studies suggest that gates may occur at some stage prior to functional synapse formation:

- 1) individual regenerating motoneurons do not innervate all muscles in the leg,

- 2) a given muscle is not innervated by all regenerating motoneurons, and
- 3) some individual motoneurons consistently fail to reinnervate certain muscles or reinnervate some muscles more frequently than others.

While these results argue for the existence of gates at some stage prior to functional synapse formation they do not enable us to decide at what particular stage the gates occur. Three possibilities are:

- 1) during axon outgrowth (stage 1, fig. 1);
- 2) after arrival at the muscle but prior to morphological synapse formation (between stages 2 and 3);
- 3) after morphological synapse formation but prior to functional synapse formation (between stages 3 and 4).

Evidence presented in the previous section argues for decision making during axon outgrowth and indeed some of the restrictions on functional innervation seem likely to be due to blockage of neuronal development at this stage; e.g. the finding that motoneuron D_f rarely regrows beyond the trochanter after crushing of nerve 5 (Denburg et al., 1977) could explain why this motoneuron rarely innervates femoral and tibial muscles (see page 53). However, other restrictions on functional innervation are not readily explained by blockage of development during axon outgrowth and point to the period between arrival of the axon tip at the muscle and formation of a functional synapse as being an important decision-making period; e.g. motoneurons 24 and 26 consistently fail to innervate muscle 137, (page 59), yet both of these neurons send axons down nerve 5r1 to the coxal depressor muscles in a high percentage of animals, (see fig. 21). The alternatives of decision-making during stage 1 or after stage 2 but prior to stage 3 could be separated by demonstrating physiologically that a particular neuron does not innervate a muscle followed by filling of that neuron with a dye which travels to the axon terminals. Evidence of dye-filled axonal terminals on the surface of the muscle in question would argue

for gates after arrival at the muscle. The same experiment may also distinguish between decision making prior to or after morphological synapse formation. Evidence of dye-filled synaptic terminals on the muscle in question would argue for gates after synapse formation (stage 3, fig. 1).

Previous work in the invertebrates and vertebrates also suggests that decisions crucial to stable synapse formation may be made prior to functional synapse formation. Where invertebrate neurons do regenerate they appear to reinnervate their original post-synaptic targets (Pearson and Bradley, 1972; Van Essen and Jansen, 1976; Palka and Edwards, 1974) whereas functional innervation of wholly inappropriate target cells is rare, see section 3.1. Several studies in the vertebrates have shown that a target cell is preferentially reinnervated by its correct nerve if both foreign and correct nerves are presented to that target at the same time (Feng et al., 1965; Sperry and Arora, 1965; Mark and Marotte, 1972; Cass and Mark, 1975). The data required to decide at precisely what stage neuron development is halted in the above cases is not available.

5.2.3 GATES AFTER FUNCTIONAL SYNAPSE FORMATION

Functional connections between regenerating motoneurons and foreign muscles were more common at early post-operative times in the nerve 5 crush study than at later times. This correction of functional "mistakes" may suggest that decisions are made after functional synapse formation: correct synapses are stabilised, foreign synapses regress. However, other interpretations are possible:

- 1) inappropriate synapses may regress not because development halts in the neuron in question but because the foreign neuron is displaced following regrowth of the appropriate neuron to the muscle. In the displacement situation decision points are indicated, not by the latest stage that the inappropriate neuron reaches, but by the level at which correct and foreign neurons compete: if, for example, these neurons compete for occupation of a limited amount of synaptic space

(see section 5.3.2.1), the "gate" occurs before functional synapse formation. Thus, while displacement may make an important contribution towards achieving specific connectivity, observations on the latest stage of development reached by a foreign neuron in this situation do not necessarily tell us anything about where gates occur.

However, if it can be established that the correct neuron has completely regrown to the muscle before the foreign neuron, displacement cannot occur and observations on the latest stage reached by an ingrowing foreign neuron may then reveal the location of gates.

Following nerve 5 crush, motoneuron D_5 appears to innervate coxal depressor muscles before 28 days post crush whereas "foreign" motoneurons (i.e. motoneurons which do not innervate coxal depressor muscles in normal animals) may take up to 43 days to establish functional connections. Yet in 72% of animals examined between 29 and 92 days muscles 135d, e, d', e' are simultaneously innervated by both D_5 and foreign neurons. In some cases dual innervation has been demonstrated at the level of a single muscle fibre. It appears likely, then, that foreign motoneurons may innervate coxal depressor muscles which are already innervated by a correct neuron. If this is the case then the disappearance of foreign synapses at later stages cannot simply be attributed to displacement.

This reasoning is open to criticism: it could be argued that in those animals in which the coxal depressor muscles are simultaneously innervated by both correct (D_5) and foreign motoneurons, that the foreign neurons had regrown faster than usual and had therefore innervated the coxal depressor muscles before D_5 . This question can only be settled by recording the connections of D_5 and foreign neurons to the coxal depressor muscles in the same animal at different post operative times. The intracellular motoneuron/muscle recording assay, the most reliable assay for connectivity, cannot be used for this purpose as the motoneuron eventually dies after being penetrated by a microelectrode: it may, however, be possible to assay neuro-

muscular connections repetitively by the extracellular recording assay method (as used in this study for motoneuron D₅; page 54).

Another interpretation of the corrections is possible. Regression of foreign synapses is dependent upon the neuron in question innervating its correct target muscles; i.e. innervation of the correct muscle provides a signal for the regression of inappropriate connections. If this "Feedback" hypothesis is correct, the correction result need not imply the existence of a gate after functional synapse formation. A test of this hypothesis is to remove the normal target muscles for a neuron and look for correction of functional mistakes made by this neuron on other muscles: if the "Feedback" hypothesis is correct, such correction should not occur.

Some recent findings in the vertebrates are consistent with the concept that crucial decisions are made after functional synapse formation, resulting in the stabilisation of these synapses.

Firstly, target cells are often innervated by more than one neuron early in development but by only one later (demonstrated in neuromuscular systems, by Bennet and Pettigrew, 1974; Brown et al., 1976 and in the central nervous system by Crepel et al., 1976). The degeneration of cortical synapses following selective sensory deprivation during a critical period in the early life of kittens (see section 1.2) may also provide an example of a stabilisation requirement in synapse development. In none of these studies, however, has the possibility that correction may be due to displacement or "Feedback" been excluded.

In summary, the present study suggests that gates occur at a number of stages in neuronal development. Development in a neuron growing to a foreign target may halt at any one of these gates, depending on the identities of the particular neuron and target. In every case, however, the result will be the same: the neuron will not form a stable, functional synapse on that target cell. Other studies in both the vertebrates and invertebrates point to the same conclusion.

It should be possible to define the decision making points for

particular neuron-target cell combinations using the approach described here. Such information will help provide a more solid base for future biochemical studies on the mechanism for specific reinnervation.

5.3 MECHANISM FOR SUPPRESSION OF ACTIVITY IN INAPPROPRIATE SYNAPSES

If functional synapses fail to be stabilised and subsequently regress, or if the correct neuron reaches a target cell after a foreign neuron has already established a functional synapse on that cell, then we require a mechanism for suppression of function in functional synapses.

It has been suggested that synapses may be functionally "repressed" without physical withdrawal of the synapse proper; i. e. synapses may exist in an anatomically normal but physiologically "silent" state. (Cass et al., 1973). The evidence on which this claim is based has recently been challenged (Scott, 1977). Nonetheless there is firm evidence to suggest that suppression of activity in functional neuromuscular synapses can be accomplished by a decline in the quantal content of junction potentials at these synapses (Yip and Dennis, 1976), a mechanism which may not require physical withdrawal of the synapse. In contrast, Bennett and Raftos (1977) argue for a reduction in size of incorrect synapses on the grounds that the value of the binomial statistic, P , which is directly proportional to synapse size in normal animals, falls as incorrect synapses become non-functional.

It was not possible to determine the fate of those incorrect functional synapses which were eliminated during correction in the present study. These synapses may have been "repressed" in the sense that they were still present in a morphologically normal but physiologically "silent" state, or they may have physically regressed. Such regression may or may not be followed by withdrawal of the axon from the nerve branch leading to the foreign muscle. The finding that fewer foreign axons are present within nerve 5r1 at 22 to 56 days post

crush compared to 64 to 222 days suggests that such axon withdrawal may occur. However, it is not known whether those axons which are withdrawn had formerly made functional connections with the coxal depressor muscles.

5.4 ROLE OF GENERAL CONSTRAINTS AND SPECIFIC CELLULAR AFFINITIES IN GENERATING SPECIFIC CONNECTIVITY

The existence of decision points during neuronal development has been inferred from restrictions on the development of regenerating motoneurons during axon outgrowth and synapse formation. It is important to determine whether these restrictions result from specific affinities or incompatibilities between pre- and post-synaptic cells, as postulated in the "Chemo-Affinity" hypothesis, or from general physical constraints.

5.4.1 AXON OUTGROWTH

Data on the frequency with which particular identified motoneurons send axons down nerve 5r1 argue for the existence of a directed axon growth mechanism. At least four mechanisms may produce such apparent directed axon growth: in the first two the axon grows out "blind" to its future target, while in the other two the axon tip recognises specific cues which guide it to its target.

1) A regenerating motor axon may simply be "channeled" down its original path since this provides the least physical resistance. Physical constraints of this type have been thought to be important in determining the path of axon growth in other systems (see page 10). Such a mechanism is clearly not operating here as regenerating motor axons may take any of several paths to reach nerve 5r1 (see page 61) i. e. they are not constrained to their original path. It is still possible, however, that physical constraints, for example amount of space available within nerve 5r1, size of axon or proximity of axons to this nerve branch, may play some role in determining how frequently an axon grows down this nerve branch.

- 2) A neuron may give instructions to its growing axon tip to send out a branch at a particular time in a particular direction (c.f. spatiotemporal models, page 10); the timing and direction of this outgrowth are such that the axon grows down nerve 5r1 even though it is growing out "blind". This mechanism is an unlikely explanation for directed growth since axonal branching distal to the crush site is often widespread. Again, however, the possibility that a mechanism of this type makes some contribution is not excluded.
- 3) The growing axon tip "reads" specific "signposts" in the nerve which direct it to nerve 5r1. "Foreign" motoneurons do not grow down nerve 5r1 as frequently as correct neurons because they do not read these signposts as well as the normal neurons.
- 4) The coxal depressor muscles release a specific diffusible chemical which "attracts" the growing axons towards them. Correct neurons respond to this attractant more readily than do foreign neurons and therefore grow down nerve 5r1 to the coxal depressors more frequently than do foreign neurons.

Either of mechanisms 3), "signpost following" or 4), "chemotropism" may provide an explanation for directed growth in the present study; they cannot be separated from available data. "Chemotropism" may, however, provide an explanation for the finding, in several other studies, that neurons can establish connections with correct targets albeit growing along aberrant pathways: "signpost following" alone is hard put to explain this result. For example, Palka and Schubiger (1975) find that sensory fibres from rotated cricket cerci can find their correct termination sites in the last abdominal ganglion. Hibbard (1967) has shown that retinal fibres may terminate at appropriate sites in the optic tectum after regenerating through the oculomotor nerve rather than the distal optic nerve root. Similarly, Sperry and Arora (1965) find that fish eye muscles may be correctly reinnervated even though the oculomotor nerves have been deflected from their original course. Studies of mutants or adult variants point to the same conclusion. Altman and Tyrer (1977) report that

branches of a locust stretch receptor neuron may take an abnormal course yet terminate at normal positions in the neuropile. The mutation "reeler" in the mouse produces abnormal cell orientations in the cerebellar cortex yet such malpositioned neurons make normal patterns of synaptic connections within the cerebellum (Caviness, 1977).

While "chemotropism" may explain the above observations there exists no direct evidence to support this theory. In fact it seems unlikely that chemical gradients could remain stable over the large distances commonly traversed by outgrowing axons. "Chemotropism" essentially exists by default as the only mechanism we can conceive of that could explain directed axon growth from misplaced neurons to their correct targets.

While mechanisms 1) to 4) discussed above represent different means of achieving directed growth, they are not mutually exclusive: the nervous system may use different mechanisms for different neurons at different stages of growth. The possible use of both specific and non-specific mechanisms by the nervous system to achieve specific connectivity is illustrated by "pathfinding" neurons: descriptive studies in the embryo (Wigglesworth, 1953; Lopresti et al., 1973; Bate, 1976b) show that, in situations where a number of neurons share the same route at least in initial stages, only the first axons to grow out "chart a course"; later neurons merely follow the pathway laid down by these first "pathfinding" neurons.

5.4.2 SYNAPSE FORMATION

What mechanism generates the restrictions on functional innervation observed in the muscle excision and nerve crush studies? These restrictions appear to be of two types: general, which apply irrespective of the identity of the neuron and may thus depend upon general physico/chemical constraints; and specific, which apply only to certain combinations of nerves and muscles and thus may result from specific differences between neurons and their targets.

5.4.2.1 GENERAL CONSTRAINTS

Restrictions of the general type suggest two basic constraints which may be important in generating specific connectivity:

- 1) Regenerating neurons may only innervate denervated muscles. This constraint appears to be a general one, having been demonstrated in a number of vertebrate studies. (Elsberg, 1917; Fort, 1940). We are ignorant of the particular property possessed by a post-synaptic cell with an intact nerve supply which enables it to resist further innervation. It is, however, known that the maintenance of this property is dependent upon continued activity in the intact nerve terminals. (Thesleff, 1960; Fex et al., 1966).
- 2) Both the muscle excision and nerve crush studies suggest that there is a limit to the number of neurons which may innervate a muscle at one time. A possible explanation of this restriction is that the space available on the muscle for synapse formation is limited. This physical constraint may be important in generating specific connectivity if correct neurons "out-compete" foreign neurons for this space.

Competition between motor nerves for the establishment of functional synaptic connections on muscle fibres has been described in several systems in the vertebrates (e.g. axolotl skeletal muscles - Cass et al., 1973; frog skeletal muscles - Grinnell et al., 1977; Haimann et al., 1976). However, it is generally unclear what the motor nerves are competing for in these examples - whether it is simply physical synaptic space or some other limiting factor. Competition for terminal space is suggested in Schneider's (1973) study of abnormal visual projections. Axons from the eye of a newborn hamster may project to the (foreign) ipsilateral colliculus if their normal target, the contralateral colliculus, is destroyed at birth. The remaining colliculus thus receives projections from both eyes. Axons from the two eyes appear to compete for terminal space in the colliculus since they terminate in a non-overlapping manner and since the extent of the abnormal ipsilateral projection increases if the axons from the normal contralateral eye are eliminated at birth.

The recent study of Donaldson and Josephson (1977) may support the suggestion from the present study that available synaptic area is also a limiting factor in neuromuscular synapse formation in the invertebrates. They find that the cricket extensor tibia muscle is normally innervated by both fast and slow axons. If the fast axon input is removed, the synaptic effectiveness of the slow axon increases. A possible interpretation of this result is that the synaptic area occupied by the slow axon is normally limited by competition from the fast axon.

5.4.2.2. SPECIFIC INCOMPATIBILITIES BETWEEN NEURONS AND THEIR TARGETS

Some restrictions on functional innervation cannot be readily explained by non-specific physical constraints of the type discussed in the previous section. In such cases we may postulate that innervation is restricted because of specific incompatibilities between the motoneuron and muscle of the type proposed in the "Chemo-Affinity" theory. As we have virtually no knowledge of the biochemical basis for such incompatibilities this hypothesis cannot be tested directly. The best that we can do at present is to eliminate all conceivable non-specific physical constraints.

5.5 CHOICES OPEN TO A NEURON - RULES FOR GROWTH AND CONNECTIVITY

It appears that, several times in development, a neuron reaches a point where it has to decide whether to continue or to stop developing. An understanding of the rules according to which this decision is made may be obtained by comparing the behaviour of a neuron, at each of these decision points, when growing to different targets. To shed light on the rules for axon outgrowth, for example, we need to set up experimental situations in which an individual motoneuron is given the opportunity of growing towards different specific muscles. Whether it accepts this offer can be tested by the orthograde or retrograde dye-

filling techniques used in this study to determine the peripheral distribution of axonal branches.

A start has been made in this study in elucidating the rules for the formation of functional synaptic connections i.e. "connectivity rules" (see pp. 13-15). As has already been noted, several conditions must be met before one can legitimately draw the conclusion "motoneuron A may not form functional synaptic connections on muscle B" from the failure to find innervation of muscle B by motoneuron A. Such conditions include ensuring that: the motoneuron has at least regrown to the muscle; the muscle offered to the neuron has been denervated; and there is available "synaptic space" on this muscle. Given such controls, it should be possible using the assay for connectivity described in this study to define the choices open to individual neurons at the stage of functional synapse formation.

5.6 BIOCHEMICAL BASIS FOR SPECIFIC CONNECTIVITY

We have virtually no information relating to the biochemical identity of the molecules responsible for generating specific connectivity in the nervous system. Clues as to the nature of such molecules may be given by the behaviour of regenerating neurons. For example, an understanding of the important decision-making stages in neuronal development may suggest where these molecules are located: molecules involved in decision-making during axon outgrowth or at the time of synapse formation are likely to lie on the cell surface whereas those involved in stabilisation of already formed synapses may be of the diffusible, trophic type (see page 8). The fact that neurons have a number of potential targets of varying degrees of acceptability may suggest that individual neurons are distinguished not by the possession of qualitatively unique molecules, but by the possession of a unique combination of a few widely occurring molecules. Finally, information on the choices open to any two neurons may suggest how chemically different we may expect these neurons to be; i.e. the degree of overlap of their range of acceptable targets may be related to how chemically

similar they are.

A biochemical search for molecules involved in generating specific connectivity has already begun. Denburg (1975) has correlated the presence of particular protein fractions from the coxal depressor muscles of the cockroach with the innervation patterns of these muscles. However, Tyrer and Johnson (1977) have claimed that these proteins correlate with whether the muscle is of the "fast" or "slow" type rather than with innervation patterns and are thus not candidate recognition molecules. What is needed to resolve this controversy is a reliable assay for molecules involved in specific recognition. Roth and co-workers (Roth et al., 1971; Barbera et al., 1973) have developed a possible assay which relies upon the strength of adhesive interactions in tissue culture between retinal and tectal neurons isolated from chick embryos. They find that neurons from a particular quadrant of the retina adhere preferentially to neurons from a region of the optic tectum which they normally innervate in vivo. If affinities of this type can be shown to be important in generating specific connectivity during development, "this assay will certainly facilitate analysis of the biochemical substrates of locus specificity through the use of inhibitors, analogues and other chemical probes" (Hunt and Jacobson, 1974).

5.7 RELEVANCE OF REGENERATION STUDIES TO DEVELOPMENT

We return now to the question of whether mechanisms for specific reinnervation deduced from regeneration studies are relevant to embryogenesis. The question can only be answered definitively by making observations on the time course of axon outgrowth and innervation of target cells by identified neurons in the embryo: some of these, for example, observations on the course of axon outgrowth appear feasible (using, for example, reconstruction from serial electron micrographs); others, for example, electrophysiological assays for functional connectivity, less so at present.

5.8 IMPORTANCE OF INVERTEBRATES IN STUDIES OF NEURONAL SPECIFICITY

Observations at the single, identified neuron level are called for if we are to obtain clear answers to the basic questions addressed in this study. For this reason invertebrates, at present, hold a considerable advantage over vertebrates as experimental animals suitable for studies of neuronal specificity. As most of the conclusions reached in the present study parallel those already reached in the vertebrates, there is considerable reason to believe that studies in the invertebrates may be of general relevance.

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